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Pathogenicity of Trypanosomes

Proceedings of a workshop held at Nairobi, Kenya, 20-23 November 1978

Editors: George Losos and Amy Chouinard

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Sponsored by
Veterinary Research Department,
Kenya Agricultural Research Institute,
Muguga, Kenya

in collaboration with
International Development Research Centre,
Ottawa, Canada,
International Laboratory for Research on Animal Diseases,
Nairobi, Kenya, and
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Foreword

This conference is the second one dealing with hemoprotozoal diseases of cattle in Africa that has been sponsored by IDRC. The first took place in December 1976 and its proceedings were published as a joint IDRC/ILRAD monograph (IDRC-086e). The present conference took place at the Intercontinental Hotel in Nairobi from 20–23 November 1978. The conference was hosted by the Veterinary Research Department of the Kenya Agricultural Research Institute at Muguga and was cosponsored by the International Development Research Centre (IDRC), Canadian International Development Agency (CIDA), and the International Laboratory for Research on Animal Diseases (ILRAD).

The objective of the conference was to review recent advances in the knowledge of the pathogenicity of trypanosomes. The main focus was African trypanosomes that are pathogenic to cattle. The conference participants sought to evaluate how information on the pathogenicity of different species of trypanosomes and on the pathogenesis of trypanosomiases could be used either to improve or to develop more efficient diagnostic and control measures. They drew attention to features that were common to all the trypanosomal syndromes and to those that constituted important differences. Emphasis was also devoted to determining how the investigations on laboratory models related, first, to experimental trypanosomiasis of livestock and, second, to the realities of the natural trypanosomiasis of humans and domestic animals.

On the 1st day, the conference was introduced by **W. Masiga**, Director of the Veterinary Research Department, and opened by **J. Muliro**, Permanent Secretary of the Kenyan Ministry of Agriculture, speaking on behalf of the Hon. J. Nyagah, Minister of Agriculture. **L. Goodwin** outlined the theme and objectives of the conference.

With **A. Njogu** chairing and **B.A. Newton** and **K. Vickerman** acting as discussion leaders and rapporteurs, the rest of the 1st day was devoted to eight papers dealing with *The Organism*, with particular reference to its structure, metabolism, biochemistry, and antigenic variation.

The 2nd day was divided into two sessions. The morning session, chaired by **S. Chema** assisted by **F.E.G. Cox** and **G.A.M. Cross** as discussion leaders and rapporteurs, contained six papers dealing with the theme of trypanosome *Infections*. After lunch, a series of five papers dealt with *Mechanisms of Cellular Injury: Blood and Circulatory System*. This session was chaired by **W. Masiga** with **P.F.L. Boreham** and **F.E.G. Cox** acting as discussion leaders and rapporteurs.

The 3rd day also consisted of two sessions. The first, chaired by **F.E.G. Cox** assisted by **J.D. Dargie** and **P.A. D'Alesandro**, comprised six papers relating to *Blood and Hematopoietic Tissue Responses*. This was followed in the afternoon by four papers covering *Lymphoid Tissue Responses* in a session chaired by **M. Murray**, with **L. Karstad** and **V.E.O. Valli** as discussion leaders and rapporteurs.

The last session entitled *Tissue Lesions* was chaired by **B.L. Nestel** assisted by **V. Houba** and **G.J. Losos**; it included four papers and concluded with a summary of the 3 days' meetings by **L. Goodwin**.

The conference was attended by more than 100 scientists from a dozen different countries, and the discussion sessions were a lively interchange of views. The organizers are particularly grateful to participants who undertook the onerous task of summarizing the discussions. These summaries appear at the end of each chapter.

All of the participants are indebted to George Losos who was responsible for the scientific planning and organization of the conference and to Bruce Scott and Mehret Ghebreyesus of IDRC whose efforts behind the scenes contributed so largely to the smooth and effective running of the meeting.

B.L. Nestel
IDRC Consultant

Welcoming address

W. Masiga

Veterinary Research Department,
Kenya Agricultural Research Institute,
Muguga, Kenya

This conference is being hosted by the Veterinary Research Department, Kenya Agricultural Research Institute, Muguga, with the financial assistance of the International Development Research Centre, the Canadian International Development Agency, and the International Laboratory for Research on Animal Diseases. The conference has drawn leading scientists from many parts of the world including Canada, France, Nigeria, United Kingdom, United States of America, Spain, Switzerland, and, of course, our own national and international research institutes. The purpose of this conference is to bring together scientists from widely distributed laboratories to discuss trypanosomiasis.

Exchange of Views Needed

Kenya is proud of the concentration of scientific talent present in the national and international research institutes in the country. Nevertheless, exchange of views is necessary, and cooperative research programs must be undertaken by research workers in this country and their colleagues abroad if there is to be rapid increase in our knowledge and control of trypanosomiasis.

The emphasis of this conference is on the exchange of ideas and the development of links for future cooperation. The laboratories abroad, in general, have better facilities, better equipment, and more financial support than those in this country. Scientists working in Kenya and the rest of Africa, however, have the advantage of living with the disease and understanding its magnitude and complexity.

The African continent has been waiting patiently for a single utopian solution to the problem of trypanosomiasis for almost a century. It is, therefore, unrealistic to expect or hope that a single universally applicable solution to the problem can be found overnight. Trypanosomiasis is a complex disease biologically, and its control is complicated by the diversity of social, economic, political, and ecologic conditions that exist on this continent. Although under certain ecologic and economic conditions trypanosomiasis can be controlled by methods that are already available, existing control measures are cumbersome, expensive, and often only temporarily effective. What is needed immediately is an improvement of the already-known methods of control through modern scientific investigations. In my opinion, this is an absolute priority, although new avenues for the control of this disease should be explored. Research resources must be realistically divided between the improvement of control methods already available and the search for new ones.

Research Directions

In summary, an attempt should be made at this conference to identify research that is to be pursued through cooperative efforts between laboratories abroad and the institutes in Africa. In essence, there should be concerted efforts around the world to fight this devastating disease. This is why we have attempted to assemble leading scientists from around the world to discuss recent advances in the knowledge of pathogenicity of trypanosomes.

Opening address

J. Muliro

Ministry of Agriculture, Nairobi, Kenya

On behalf of the Kenyan Government and the Ministry of Agriculture, I would like to welcome you all to this conference, which is being hosted by the Veterinary Research Department of the Kenya Agricultural Research Institute, Muguga. I extend my welcome to the participants from abroad who come from the various continents including North America, Europe, and Africa, and to the participants from our own national and international institutes.

It gives me great pleasure to welcome you to Kenya, and particularly to Nairobi, the City in the Sun. I think that those of you coming from temperate regions will find our present weather a little more friendly than what you are experiencing at home. I hope that during your stay in our country you will not be confined totally to deliberations in this conference but that you will take the opportunity to see more of Nairobi and, possibly, the other regions of Kenya.

The Ministry of Agriculture attaches great importance to this conference because it deals with major problems of the livestock industry. Agriculture is the backbone of Kenya and other countries in East Africa, and animal production plays a major role in the feeding of our people.

One of the priorities given in the development plans of the Ministry of Agriculture is the livestock industry. The importance of livestock is clearly evident when one realizes that only about 15% of the land area of Kenya is prime arable land. The rest of the country has relatively low rainfall and is suited primarily for rearing of livestock. In this respect, Kenya is representative of the availability of arable and pasture land of many of the countries in East Africa.

One method by which productivity in the livestock industry can be increased is through better control of animal diseases. In the past, Kenyan institutes have played a leading role in developing methods for the control of the economically important animal diseases. These control methods are now being utilized throughout the world. With this history, it is appropriate that Kenya should again focus on research to control the remaining diseases, especially those caused by tsetse flies, which still plague the entire continent of Africa. The Kenya veterinary profession has a long record of contributing significantly to livestock development. The Kenyan Institute, which is hosting this conference, has played a central role in the development of vaccines — the major achievements being the development of a vaccine against Rinderpest and one against Contagious Bovine Pleuropneumonia.

It is now time to consider the economic importance of other diseases such as trypanosomiasis. It is hoped that this conference will encourage exchange of new information and will provide contacts between Kenyan institutes and research institutes and organizations in other parts of the world so that progress can be made quickly to improve control of a disease that plagues the cattle populations of Kenya and Africa. Kenya and other countries in Africa must increase their food productivity to keep pace with the growing populations. Because of their climatic problems, such as scarcity of rainfall, the countries must focus on animal production and explore means by which the production of cattle, sheep, goats, poultry, camels, and game animals can be increased.

We, therefore, hope that during your deliberations of the next few days you will be able to understand the problems facing the livestock industry in Kenya and in Africa and through your joint knowledge and expertise in this field you will point the way to the control of trypanosomiasis.

In conclusion, I wish you a successful conference and an interesting and enjoyable stay in Nairobi; I extend to you the appreciation of the Minister of Agriculture for your participation in this important scientific conference.

Vote of thanks

B.L. Nestel

International Development Research Centre, Ottawa, Canada

I have been asked on behalf of the overseas visitors to this conference to express our appreciation through the Permanent Secretary to the Minister of Agriculture and to his Government for making this meeting possible.

For some years now, Kenya has been a very active centre of research on trypanosomiasis. As can be observed from the number of local participants here today, there is a large population of scientists in different disciplines working in both national and international institutes, who are based in Kenya and are researching trypanosomiasis and its tsetse fly vector. It would be difficult to find anywhere else in the world where one could so readily bring together so much expertise on the theme of the pathogenicity of trypanosomes.

The Veterinary Research Department of the Kenya Agricultural Research Institute directed by Walter Masiga, our chairman this morning, is responsible for bringing us here today. This meeting offers an opportunity for a dialogue between those working in Kenyan-based institutes and other distinguished workers throughout the world.

On behalf of the two Canadian institutions, I would like to say how happy we are to be associated with the Kenya Agricultural Research Institute in the organization of this conference. It is gratifying to see that a conference of this nature has attracted specialists from Nigeria, France, Canada, Holland, West Germany, Switzerland, the United Kingdom, and the United States, and has drawn upon expertise on American trypanosomes as well as on African ones.

Organizing a conference of this nature involves a lot of hard work, and Dr Masiga and his team at Muguga are to be complimented on the groundwork that they have laid for what promises to be a stimulating 3 days.

We would not have had the opportunity to be here today if the Veterinary Research Department and the Veterinary Services Department of the Government of Kenya did not receive such strong and consistent support from the Minister of Agriculture and his Permanent Secretary under whose leadership the livestock industry of Kenya has made such significant progress.

The recent history of livestock development in many African countries shows that it is no mean task to move the industry forward to the extent that has occurred in the last decade in Kenya. The industry is fortunate in having a Minister and a Permanent Secretary who take such an active interest in its progress and play such an active role in its development.

Theme and objectives of the conference

L. Goodwin

Nuffield Institute of Comparative Medicine, London, England

It is a pleasure to launch this conference on recent advances in the knowledge of pathogenicity of trypanosomes because it is a subject that has intrigued me for many years. It has always been something of a mystery to me to see an animal afflicted with nagana eat steadily away and, at the same time, waste steadily away to skin and bone and perish with, at first glance, little to show at postmortem.

A closer look reveals a deal of mischief, acutely observed and accurately described by David Livingstone 120 years ago. He knew that nagana was carried by tsetse, although he never saw the parasite. In his *Popular account of missionary travels and researches in South Africa*, published in 1861, he gives an illustration of the fly and says: "... its bite is death to the ox, horse and dog. In this journey, though we watched the animals carefully and believe that not a score of flies were ever upon them, they destroyed 43 fine oxen."

Livingstone's African friends also knew all about it, long before. The Chief, Sebituane, welcomed him with joy and added, with characteristic African generosity: "Your cattle are all bitten by the tsetse and will certainly die; but never mind, I have oxen and will give you as many as you need."

Livingstone describes with admirable accuracy the way the fly bites, the progress of the disease in infected cattle and the postmortem appearances:

> The blood is small in quantity and scarcely stains the hands in dissection .... All the muscles are flabby and the heart is often so soft that the fingers may be made to meet through it .... These symptoms seem to indicate poison in the blood; the germ of which enters when the proboscis is inserted.

It would be difficult to do better than that — low blood volume, severe anemia, tissue destruction, and toxic substances in the blood.

It will be the object of this conference to discuss interactions between the host and the parasite that cause the disease. Infections do not necessarily result in disease — all of us are infected with countless microorganisms that do no harm; the only animals entirely free from infection are the cesarean-derived, germ-free creatures that live expensively in isolators.

Well-adapted parasites do little harm to their normal hosts and the principle of "live and let live" has been thoroughly worked out by trypanosomes over the millenia. *Trypanosoma congolense* and *T. vivax* in adult African wild animals, *T. brucei* in cattle, and *T. cruzi* in opossums and armadillos need not seriously incommode their mammalian hosts. However, the balance is sometimes precarious, and symptomless infections may be precipitated into fatal disease by stress, climatic or nutritional adversity, or intercurrent infections.

At this conference, we propose to look in detail at the characteristics of trypanosomes, the diseases they cause, their hosts' responses, and the damage that results in cells, tissues, and organs. We aim to focus our discussions on the similarities and differences that exist between the forms of trypanosomiasis caused by different species of trypanosome, and we shall try to assess the value of laboratory tests as models of the disease in humans and domestic animals.

We have, in fact, come a long way in our understanding of the complex process since Livingstone's time, and the organizers of this conference have brought together an impressive team of active research workers to assess our present knowledge and to point the way ahead.

The success of the conference will depend on the discussions that take place, in this room and outside it, and we invite you all to make full use of the opportunities provided to put forward your findings, your ideas, and your fantasies.

The metabolism of African trypanosomes in relation to pathogenic mechanisms

B.A. Newton

Medical Research Council Unit for Biochemical Parasitology, The Molteno Institute, University of Cambridge, England

Abstract. The metabolism of a parasite might affect its host in two ways: by depleting essential nutrients and/or producing toxic metabolites. The possibility that glucose consumption, pyruvate production, and the deamination of the amino acids tyrosine and tryptophan might be important in the effects of African trypanosomiasis is examined in the light of recent work.

In a recent discussion on the mechanisms of pathogenesis in African trypanosomiasis, Goodwin (1974) stated: "The pathogenesis is complex and the cause of death is still somewhat obscure. Damage to the tissues is brought about perhaps through the metabolic activities of the trypanosomes, more certainly through the repeated insults offered by the emergence of successive trypanosome variants and the attempts made to suppress them by the hosts' defence mechanisms." Our discussions at this conference will centre largely upon the nature of these "insults" and with the way the host responds to them, but it is my task in the next 30 minutes to consider the "perhaps" clause of Goodwin's comment. Thirty minutes is of course an impossibly short time in which to review present knowledge of trypanosome metabolism — a recent review of oxidative metabolism (Bowman and Flynn 1976) ran to more than 30 pages; on the other hand, it is a dauntingly long time in which to discuss the metabolism of these parasites in relation to their pathogenicity where, all too soon, one enters the realm of speculation. Thus, of necessity, this paper will be something of a compromise between the two. Clearly, the metabolic activity of a parasite might affect its host in two ways: by depleting essential nutrients and/or producing toxic metabolites. These two possibilities have been discussed in relation to trypanosomiasis for more than half a century, but we still lack firm evidence that either plays a key role in pathogenicity and in recent years attention has focused more on immunologic reactions and the release (from host tissues or damaged parasites) of pharmacologically active substances than on the effects of trypanosome metabolism. However, I am sure it is premature to dismiss the metabolic activity of trypanosomes as unimportant in pathogenesis — we still know far too little about the subject to do that.

Studies on the physiology and biochemistry of trypanosomes have tended to centre on carbohydrate metabolism and, in particular, on the changes in oxidative pathways associated with the developmental cycle of the *T. brucei* group (Newton, Cross, and Baker 1973; Vickerman and Preston 1976). The story that emerged is too well known to warrant detailed discussion here: the difference in cyanide sensitivity between blood and culture forms, the development of a functional cytochrome system in the latter, the differences in the end products of glucose metabolism, and the presence of the glycerophosphate oxidase pathway in blood forms are all well documented and have been the subject of several excellent reviews (see for example Fulton 1969; von Brand 1973; Bowman and Flynn 1976). In terms of pathogenic mechanisms, these aspects of metabolism seem singularly unpromising because, to quote Goodwin (1974) once more, "...none of the metabolites along the several pathways available are recognizable as dangerous poisons." However, one of the earliest, and for 30 years one of the most controversial, hypotheses on the cause of death in trypanosomiasis stemmed from the observation that the motility of trypanosomes in blood depends upon

adequate supplies of glucose (Schern 1928). It was suggested that so much glucose was consumed that the hosts' carbohydrate reserves became exhausted resulting in a breakdown of liver function and the onset of lethal hypoglycemia. This view was accepted by many workers, and evidence in support of it was published as recently as 1956 (reviewed by von Brand 1973); however, as von Brand points out, this hypothesis is not easily reconciled with a number of observations:

- Blood sugar levels return to normal, even in fasting animals, after administration of trypanocidal drugs (Scheff 1932).
- Liver glycogen reserves, although lowered, are not eliminated in infected animals (Mercado and von Brand 1960; Marciacq and Seed 1970; Lumsden, Merciacq, and Seed 1972; Ashman and Seed 1973).
- Feeding glucose to infected animals may prolong their lives for short periods but does not prevent the onset of terminal hypoglycemia (Andrews, Johnson, and Dormal 1930; Hoppe and Chapman 1947).
- The major end-products of glucose breakdown by trypanosomes are pyruvate and glycerol, which are readily metabolized by the host to produce a considerable amount of energy.

In the light of these and other facts, von Brand (1973) suggested that terminal hypoglycemia in trypanosomiasis is more likely to be due to a breakdown in hepatic or endocrine mechanisms controlling the mobilization of carbohydrate reserves than a direct result of the massive consumption of glucose by trypanosomes. However, it may be premature to dismiss this metabolic activity of the parasites as being of no consequence to the host: Voorheis (1969) has suggested that the continual demand for glucose by the parasite in acute infections may result in decreased glucose metabolism in the hosts' peripheral tissues leading to a condition resembling diabetes mellitus. This idea merits further investigation.

Another aspect of the high glucose consumption by bloodstream trypanosomes is the production of pyruvate and how it relates to pathogenesis. Although pyruvate is readily used by host tissues, it has been shown in laboratory infections to accumulate in the blood in amounts directly proportional to the numbers of parasites present (Grant and Fulton 1957; Coleman and von Brand 1957). High concentrations of pyruvate could lead to depletion of alkali reserves, acidosis, and a lowered affinity of hemoglobin for oxygen. Naus and Yorke (1911) drew attention to the dark purple

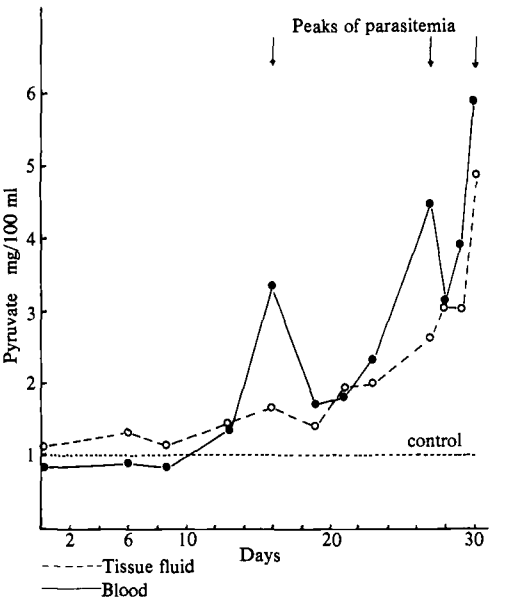

Fig. 1. *Effect of* T. brucei *infection on blood and tissue fluid pyruvate levels in rabbit (from Goodwin 1974).*

colour of blood in late stages of *T. brucei* infections, and Andrews, Johnson, and Dormal (1930) proposed that deficient oxygenation of hemoglobin coupled with mechanical blockage of the circulation leads to death by asphyxiation. Coleman and von Brand (1957), on the other hand, conclude that pyruvate does not reach a generally toxic level in the bloodstream. It must be stressed, however, that the *T. brucei* group, which are humoral rather than hematic parasites (Losos and Ikede 1972), may very well produce toxic levels of pyruvate at extravascular sites. Goodwin and Guy (1973) using their elegant "hair-curler technique" have studied pyruvate levels in blood and tissue fluids of *T. brucei*-infected rabbits and have found increases to five times the normal value (Fig. 1). They suggest that the high concentrations of pyruvate in tissue fluid may be associated with the observed changes in the structure of connective tissues covering the subcutaneously implanted hair curlers. In control animals this connective tissue, which is composed mainly of collagen fibres, fibroblasts, and blood vessels, is smooth, well organized and free from lipid, whereas in *T. brucei*-infected animals it has a rough surface. Moreover, the fibroblasts in infected animals contain lipid droplets, cease to produce collagen fibres, and may become detached into the surrounding tissue fluid (Goodwin, Guy, and Brooker 1973). Discussing these results, Goodwin (1974)

points out that although fibroblasts normally produce and store lipids, they rarely exhibit large lipid droplets in cytoplasm (Noble and Boucek 1955). Fibroblasts cultured in the presence of excess fatty acids do accumulate such droplets. Thus, it seems possible that the high in vivo concentrations of pyruvate, which occur in *T. brucei* infections, produce a similar effect on fibroblasts and are responsible for, or at least contribute to, the degenerative changes observed in the connective tissues of infected rabbits. In considering this possibility, it is interesting to note that infections with hematic trypanosomes (*T. congolense* and *T. vivax*) are not characterized by extensive inflammatory, degenerative, and necrotic changes and that these organisms do not produce as much pyruvate per mole of glucose metabolized as do *T. brucei* group trypanosomes (Fig. 2).

Compared with our knowledge of carbohydrate metabolism, we know relatively little about the metabolism of amino acids by hematozoic trypomastigotes. Tracer experiments have shown that alanine is the major amino acid produced from glucose by *T. rhodesiense* and *T. gambiense* (Grant and Fulton 1954; Shaw, Voller, and Bryant 1964; Chappell, Southworth, and Read 1972). A number of other amino acids are also labeled (aspartate, glutamate, glycine, and serine), and there is evidence that trypanosomes can interconvert some of them, but there is no estimate of the proportion of the total amino acid requirement for growth that is satisfied by *de novo* synthesis. Exogenous amino acids are known to enter trypanosomes by both diffusion and specific transport systems (Voorheis 1973; Jackson and Fisher 1977), and there is evidence that blood forms of *T. brucei* are capable of ingesting and digesting proteins (Langreth and Balber 1975). Again, the relative importance of these processes in satisfying the organism's requirements is unknown. It has been suggested (de Raadt and Seed 1977) that the availability of ingestible proteins, amino acids, and other nutrients is an important factor in determining when, during an infection, trypanosomes begin to develop in the cerebrospinal fluid (CSF). The parasites may have access to the CSF throughout an infection (Peruzzi 1928b) but be unable to grow until it has been enriched by proteins and other nutrients from degenerating tissues.

How much stress to the host's nitrogen metabolism results from the parasites' demand for amino acids is unknown. Serum and tissue fluid albumin levels fall steadily during the course of a *T. brucei* infection in rabbits, and levels of nonprotein nitrogen (particularly proline, alanine, creatinine, and urea) rise (Goodwin and Guy 1973). A detailed

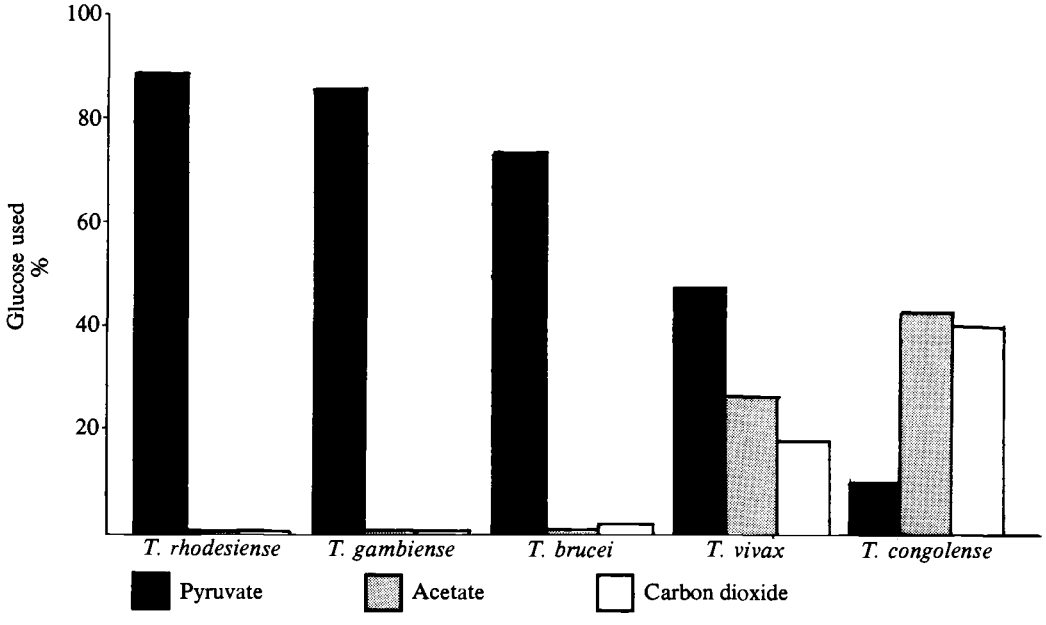

Fig. 2. *Pyruvate, acetate, and carbon dioxide resulting from the metabolism of glucose by blood forms of hematic and humoral trypanosomes (from Ryley 1956).*

Table 1. Serum and hepatic tyrosine aminotransferase activity in uninfected and *T. gambiense*-infected voles.

Group	Serum (nmoles substrate/mg protein/h)	Hepatic (μmoles substrate/mg protein/h)
Control	0	2.29
T. gambiense-infected	17.66	5.43

Source: Stibbs and Seed (1976).

study of free serum amino acids in voles (*Microtus montanus*) infected with *T. gambiense* has also revealed major changes (Newport et al. 1977). In control animals, the majority of amino acids showed diurnal variation, levels being highest during the dark period; this pattern was not found in infected animals and the levels of 7 (threonine, serine, valine, isoleucine, leucine, tyrosine, and tryptophan) of 18 amino acids studied fell significantly below the levels in the controls. In agreement with Goodwin and Guy's (1973) rabbit experiments, it was found that alanine and proline levels were markedly increased at certain stages of the infection. Of the amino acids that were reduced, tyrosine and tryptophan were most affected: tyrosine to about 50% of control levels and tryptophan to undetectable levels. The fall in tyrosine was predicted by Stibbs and Seed (1976) when they found elevated serum and hepatic tyrosine aminotransferase levels in *T. gambiense*-infected voles (Table 1). They point out that tyrosine metabolism has seldom been investigated during a parasitic infection, although this amino acid is an important precursor of catecholamines (Fig. 3). Goodwin (1970) drew attention to the fact that norepinephrine alleviates the shock that accompanies protozoal infections and suggested that catecholamine metabolism is defective in African trypanosomiasis; the work of Stibbs and Seed (1976) and Newport et al. (1977) seems to support this idea given that in other mammalian systems a fall in serum tyrosine, relative to other neutral amino acids, restricts tyrosine transport across the blood-brain barrier and lowers derivative catecholamine pools in the brain (Wurtman et al. 1974). In keeping with these findings, Newport and Page have found a reduction of 32–45% in brain, liver, and skeletal muscle tyrosine in *T. gambiense*-infected voles (Table 2). Speculating on the significance of these results, Stibbs and Seed (1976) suggest that a reduced brain tyrosine level accounts for some of the neurological syndromes occurring in *T. gambiense* infections; it is known that depression of catecholamine biosynthesis results in changes in sleep or activity patterns (Jouvet 1969), body temperature (Svensson 1971), glycogen, and lipid metabolism and possibly causes mental depression (Schildkraut 1965). Similarly, it is possible that the observed fall in serum tryptophan (Newport et al. 1977) could result in decreased synthesis of niacin and serotonin by the host, leading to a pellagra-like syndrome, changes in sleep patterns, and depression (Stibbs and Seed 1975a). At present, we cannot judge just how much of the reduction in serum tyrosine and tryptophan is

Table 2. Tyrosine levels in brain, liver, and muscle tissue from uninfected and *T. gambiense*-infected voles.

Tissue	Control	*T. gambiense*-infected
Brain	0.107	0.059
Liver	0.265	0.181
Muscle	0.187	0.103

Source: Newport and Page (1977).

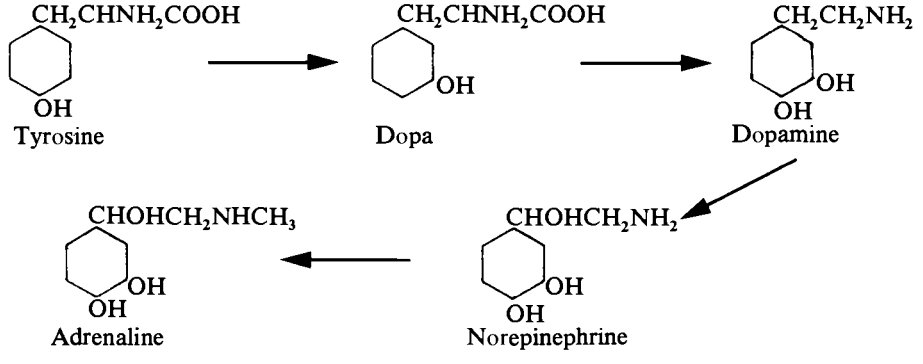

***Fig. 3**, Metabolic pathway for catecholamine biosynthesis from tyrosine.*

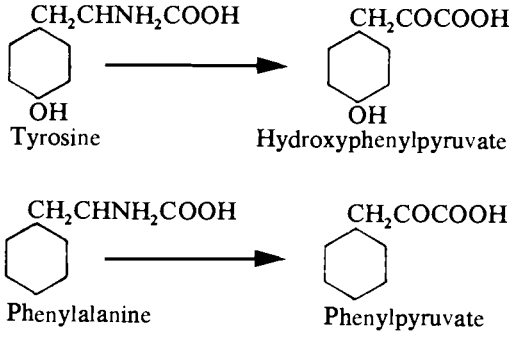

Fig. 4. *Deamination products of tyrosine and phenylalanine.*

due to the metabolic activity of viable trypanosomes, changes in host metabolism, or the release of enzymes from degenerating cells; the work of Stibbs and Seed (1976) suggests that tyrosine aminotransferase is released from trypanosomes after agglutination and lysis by variant-specific antibody. We do know that both tyrosine and tryptophan are actively metabolized by *T. gambiense*, so it seems reasonable to discuss these effects under the title of this lecture.

There is increasing evidence that the accumulation of end-products of amino acid metabolism contributes to the characteristic pathology of African trypanosomiasis. Transamination of tyrosine yields p-hydroxyphenylpyruvate (Fig. 4), high concentrations of which have been found in the urine but not in the blood of infected animals (Stibbs and Seed 1975c, 1976). This metabolite is closely related to phenylpyruvate (formed by transamination of phenylalanine), which is a known inhibitor of adrenaline synthesis. Accumulation of either of these deamination products in the brain could contribute to the pathological picture of Gambian sleeping sickness. Clearly, this is an area demanding further research. Similarly, deamination of tryptophan yields pharmacologically active compounds, and considerable interest in these has been aroused by the work of Seed's group. Indole pyruvate, the immediate product of tryptophan transamination, is further metabolized to indole lactate, indole acetate, and indole ethanol (tryptophol) (Fig. 5) (Stibbs and Seed 1973, 1975a, b, and d). There is evidence that tryptophol can cause sleep, convulsions, and death by respiratory depression when injected into mice, rats, and cats (Sabella et al. 1969), and it has been suggested that trypanosomes in extravascular sites of the central nervous system produce sufficient quantities of this indole to produce similar effects. Tryptophol has also been reported to cause immunodepression in laboratory rodents (Ackerman and Seed 1976) and may contribute to it in humans and other animals. It is thought that tryptophol acts on cell membranes, perhaps by combining with the outer lipid bilayer (Tizard et al. 1979), and in support of this, recent

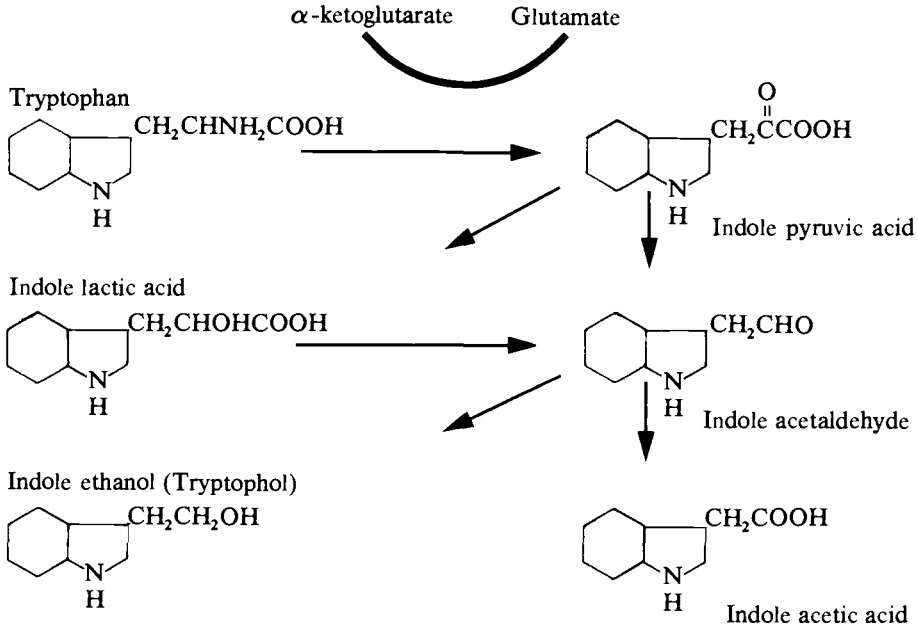

Fig. 5. *End products of tryptophan metabolism by* T. gambiense *(from Stibbs and Seed 1975a).*

work (Seed, Seed, and Sechelski 1978) has shown that tryptophol rapidly lyses red blood cells. Similar action on synaptic membranes may cause changes in the transmission of nerve impulses, give rise to behavioural changes, and induce a sleep-like or comatose state.

An essential step in testing the hypothesis that tryptophol produced by trypanosomes is important is the determination of in vivo levels of the metabolite during infection and the correlation of these with the various states observed. This work is in progress in Seed's laboratory. As a first step, Seed and Hall (1977) have estimated from in vitro measurements that a minimum of 3.2 mg tryptophol/kg body weight could be formed in an infected mouse. This level is, they believe, compatible with their hypothesis, which is further supported by the finding that levels of indole lactate and indole acetate in the urine of trypanosome-infected mice are about three times those found in control animals.

It is not yet possible to say from any of this work that tryptophol or other metabolites of aromatic amino acids are responsible for the abnormal behavioural and/or pathological changes observed in infected animals, but the investigations by Seed and his collaborators have opened up a whole new area of research that may lead to an explanation of at least one aspect of African trypanosomiasis. Mental disturbances are often found (80–95%) in chronic human infections (Lambo 1966); histological studies have thrown no light on their cause, and the behavioural syndromes of the disease remain a clinical mystery. Perhaps the answer will come from detailed studies of trypanosome metabolism and investigation of the pharmacological activity of the metabolites they produce.

Acknowledgments

I wish to express my gratitude to L.G. Goodwin for the loan of slides and to J.R. Seed for providing preprints of recent work and for allowing me to quote unpublished results.

Biology and ultrastructure of trypanosomes in relation to pathogenesis

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Abstract. The powerful locomotory flagellum and microtubule-reinforced body of the trypanosome endow it with the ability to inflict mechanical damage on its host by penetrating through or between cells. Pinocytosis from the flagellar pocket deprives the host of plasma proteins. Destructive lysosomal enzymes and the residual bodies of autophagosomes are discharged into the flagellar pocket. Antigen–antibody complexes, which are believed to be at the root of much trypanosome pathogenesis, are produced when a host's antibody combines with either common antigens, which are released from residual bodies and from lysed trypanosomes, or variable antigens, which compose the surface coat of the trypanosome. The ability to replace one surface coat with another (antigenic variation) made of a different glycoprotein antigen enables the salivarian trypanosomes to avoid the host's immune responses.

We shall here consider the trypanosome as a destructive piece of machinery, examining the working of its parts in relation to the different ways in which it harms its host. This parasite deprives its host of essential substances while burdening it with unwanted wastes; it inflicts mechanical damage through its incessant physical activity; and it goads the host into responding to its presence and then evades the backlash. These activities will be set against the background of trypanosome biology.

Trypanosomes in Mammals

In cyclically transmitted trypanosomes (i.e., where the parasite has undergone multiplication and development in the vector), infection of the mammal is initiated by the metacyclic stage, the trypanosomes being injected with the saliva of the tsetse fly (Salivaria) or contaminating the ruptured epidermis from vector hindgut contents (Stercoraria). In the tsetse-borne trypanosomes, the metacyclic trypanosomes develop further in the chancre that forms in the host's skin at the site of the bite. *T. brucei* metacyclics transform into long thin slender forms that invade the local lymph vessels and then the bloodstream where they multiply by binary fission. From the vascular system the trypanosomes may invade the connective tissues. Waves of trypanosome multiplication are detectable in the host's blood, the peak and remission of each wave being characterized by an increasing percentage of nondividing short stumpy forms. Unlike the slender forms, the stumpy trypanosomes and those of intermediate morphology are poorly infective to other mammals by syringe transfer but if ingested by the tsetse fly will initiate the cycle in the vector. The differences in infectivity accompanying trypanosome pleomorphism correlate with changes in the trypanosome's single mitochondrion and correlated energy metabolism (Vickerman 1965, 1971). The stumpy forms partially activate the repressed mitochondrion and so become preadapted to life in the tsetse fly, where proline and other amino acids replace glucose as the parasite's principal source of energy.

Chronic infections in the mammal are made possible by antigenic variation. Each parasitemic wave represents a population composed of trypanosomes carrying a different surface antigen from those of the previous wave. Parasitemic remission is induced by the host's immune response (Vickerman et al. 1976) to a particular variable antigen type (VAT). The stumpy forms are more resistant to immune assault than their slender progenitors (Balber 1972), but the small minority of slender forms that bear a different surface antigen — the heterotypes — evade the immune system's attack altogether and survive to continue the infection. We do not know what halts trypanosome division and

induces genesis of the stumpy forms, but the transformation occurs in the absence of a detectable immune response (Balber 1972; Vickerman et al. 1976).

In *T. vivax* and *T. congolense*, pleomorphism and distinct dividing and nondividing stages may be present in the mammal host (Nantulya et al. 1978), but mitochondrial changes have not yet been detected. Both trypanosomes are believed to be hematic, i.e., confined to the vascular system and not prone to invade secondarily the connective tissues (Losos and Ikede 1972). *T. congolense* certainly has an extensive primary developmental phase in connective tissue of the chancre (Luckins and Gray 1978), with distinct morphological forms (reminiscent of the fly proventricular stage) (see Roberts, Gray, and Gray 1969). The bloodstream trypanosomes are attached to the endothelium of small vessels (Banks 1978; Büngener and Müller 1976) according to a circadian rhythm (Hawking 1978). *T. vivax* is not known to be localized in this way. Antigenic variation is a feature of infections with both species.

Pleomorphism is rare in the mechanically transmitted *T. evansi* and unknown in the venereally transmitted *T. equiperdum*, which is almost exclusively a parasite of the connective tissues in equines. Evidence from morphology and geographical distribution suggests that both are descendants of *T. brucei* (Hoare 1972); like the parent species both undergo antigenic variation.

In contrast, stercorarian trypanosomes illustrate the alteration of dividing and nondividing phases. *T. lewisi* undergoes multiple fission in the epimastigote form lodged in capillary vessels for 5 days following infection by metacyclics. Nondividing broad trypomastigotes are released into the blood but shortly afterwards are usually killed by a trypanolytic antibody. A few of the trypomastigotes resist the attack and survive to infect the vector, but they are later demolished by a second trypanolytic antibody. The peculiar immunoglobulin ablastin (see D'Alesandro p. 63) is responsible for checking division of *T. lewisi* in the blood, and, as in *T. brucei*, respiratory and mitochondrial changes accompany the gross morphological changes of the trypanosome (reviewed by Vickerman 1971).

Whereas *T. lewisi* is capable of limited antigenic variation (implicit in the failure of the first lytic antibody to erase all trypanosomes), *T. cruzi* does not appear to have the capability at all, having a pattern of development that renders variation unnecessary. In *T. cruzi*, division takes place in intracellular amastigotes in muscle, mononuclear phagocyte or nerve, sheltered from the host's immune assault. Nondividing trypomastigotes are released into the blood from the ruptured host cell. Although slender and broad forms have been observed among bloodstream forms of *T. cruzi*, evidence that the former may carry on the infection in the mammal and the latter serve to infect the vector (Brener 1976), paralleling the situation in *T. brucei*, is insufficient at present. Strangely, respiratory differences between the different stages in its life cycle are quantitative rather than qualitative (Gutteridge, Cover, and Gaborak 1978).

Demonstration of a common basis to the life cycles of the trypanosomes of mammals is still lacking. The claim that *T. brucei* has an "occult" dividing amastigote (or spheromastigote) stage in the mammalian host during which antigenic variation occurs (Ormerod and Venkatesan 1972) has not been confirmed. The brain-inhabiting forms of *T. brucei* that survive chemotherapy (Jennings et al. in press) may simply be slender trypanosomes located beyond the blood-brain barrier. The amastigote and multinucleate flagellates may actually have little role in the life cycle, as they are frequently the products of degeneration or aberrant morphogenesis. Ruthless selection of viable forms is probably a feature of all protozoan life cycles.

Functional Anatomy of a Trypanosome

The principal morphological features of a generalized salivarian bloodstream trypanosome are revealed by the electron microscope (Fig. 1 and 2). Cortical (pellicular) microtubules, longitudinally disposed, lie immediately beneath the plasma membrane of the flagellate's body and maintain its elongate shape. The single flagellum arises from a basal body (kinetosome) submerged in the floor of the flask-shaped flagellar pocket. After leaving the neck of the flask, the flagellum is attached to the body between two cortical microtubules to become free at the flagellate's anterior extremity; there is no projecting free flagellum in *T. congolense* nor in the advanced stumpy form of *T. brucei*. The single mitochondrion is associated with the locomotor apparatus through a capsular expansion of the former which houses the kinetoplast (Fig. 2).

The trypanosome's ability to move and its ability to alter its shape go hand in hand. Beating of the flagellum, and hence propulsion of the trypanosome, may proceed in either direction but usually the waves pass from tip to base of the flagellum; hence the flagellar tip marks the organism's anterior end. A series of junctional complexes of the desmosome type (see Brooks 1978) attach the flagellum to the body and the body is deformed by the beating flagellum to give the characteristic undulating membrane appearance. Powerful swimming movements combined with the lon-

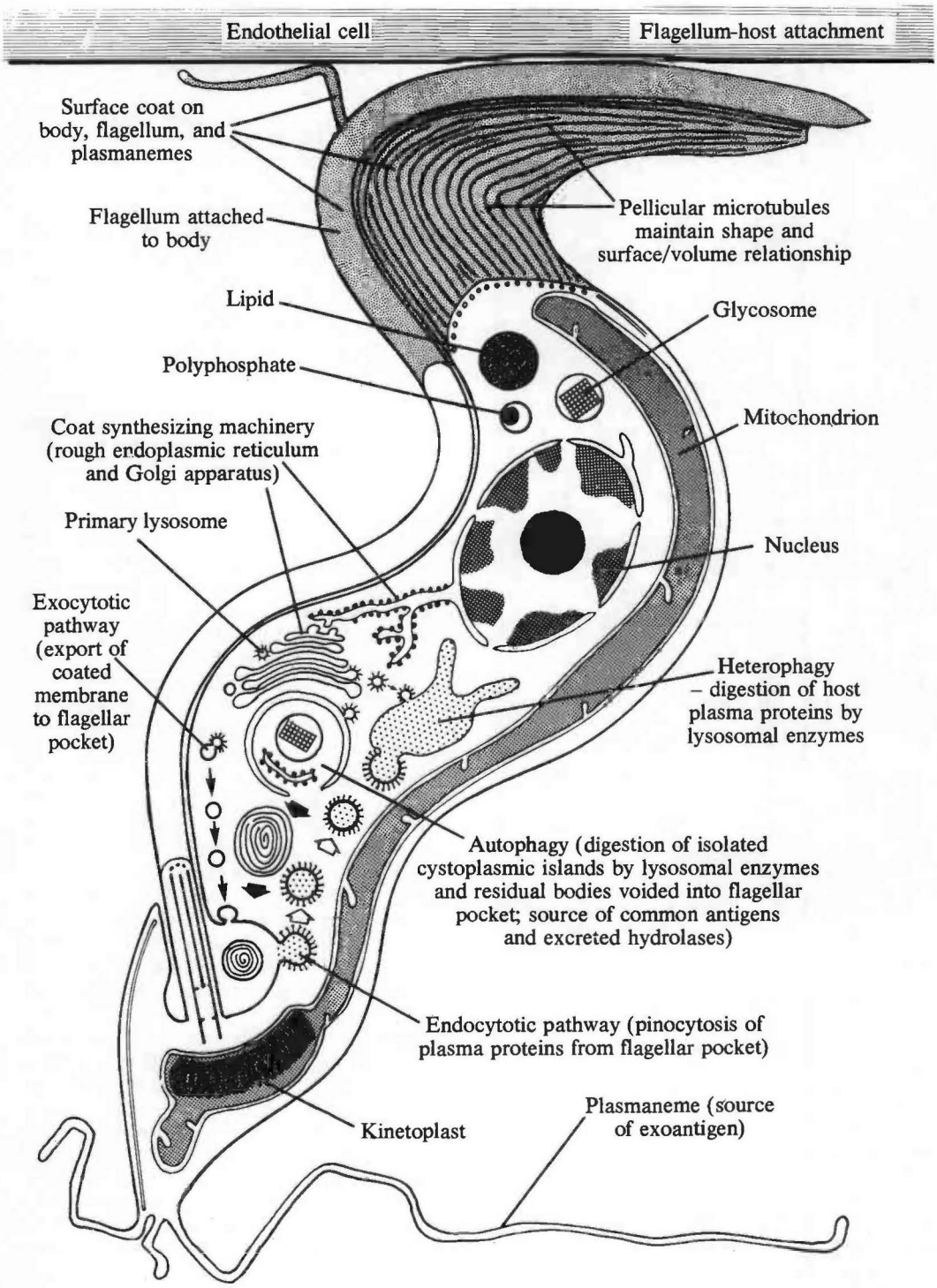

Fig. 1. *Principal structures of a salivarian trypanosome and their functions in relation to pathogenesis.*

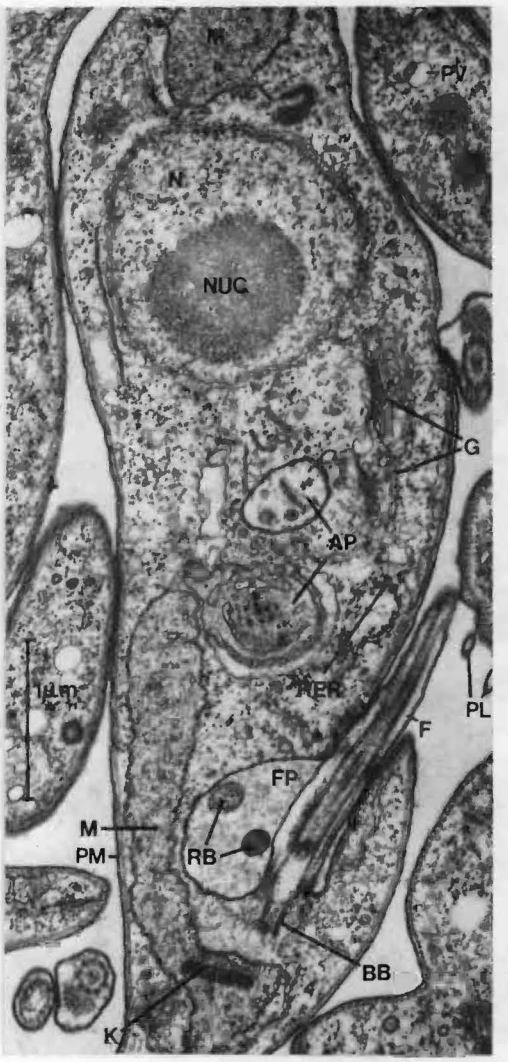

Fig. 2. Electron micrograph of longitudinal section of posterior half of stumpy bloodstream form of T. brucei: *the flagellum (F) arising from the flagellar pocket (FP), its basal body (BB) apposed to the kinetoplast (K)-bearing region of the single mitochondrion (M), the nucleus (N) with its large nucleolus (NUC), rough endoplasmic reticulum (RER), the Golgi apparatus (G), and partially formed autophagosomes (AP). The body is supported by pellicular microtubules (PM), the flagellar pocket contains exocytosed residual bodies (RB). Adjacent trypanosome profiles show a polyphosphate vacuole (PV) and plasmanemes (PL). Fixation in glutaraldehyde, followed by osmium tetroxide, staining with uranyl and lead.*

gitudinal reinforcement of the slim body by pellicular microtubules and lateral deformability enable the trypanosome to penetrate between cells and into cells. Details of how penetration is effected, however, are surprisingly sparse. Whether in their migratory activities in the mammalian body trypanosomes can split apart junctional complexes binding endothelial cells, actively penetrate the endothelial cell membrane, or induce the cell to phagocytose and then exocytose them, is not known. *T. brucei* procyclics appear to invade *Glossina* midgut epithelial cells (Evans and Ellis 1978) by active penetration. *T. cruzi* blood trypomastigotes induce even "nonprofessional" phagocytes to engulf them (Nogueira and Cohn 1976), and the ability of *T. cruzi* actually to penetrate cell membranes (e.g., that of the parasitophorous vacuole) is well established (Kipnis, Calich, and DaSilva 1979).

One unique feature of the trypanosomatid flagellum is its ability to form attachments to host surfaces. The ultrastructural details of the attachments to the chitin-lined regions of the vector's gut are now well known (reviewed by Molyneux 1977). Not so well characterized is the attachment of *T. congolense* to the walls of small (10–30 μm diameter) blood vessels (Banks 1978). Electron micrographs of the attachment (Büngener and Müller 1976) have not as yet revealed the characteristic hemidesmosome-like arrangement of filaments (Vickerman 1973) converging on the flagellate's membrane in the attachment region, but interdigitations of the membranes of parasite and host have been reported. Attachment of trypanosomes to phagocytic cells also seems to be mediated initially by the flagellum (Stevens and Moulton 1978; Takayanagi, Nakatake, and Enriquez 1974). Macrophage cytophilic antibody induces attachment in vitro, but opsonizing antibody is necessary for engulfment of the parasite (Vasquez, Cattan, and Herbert 1975).

When attached to host surfaces, the trypanosome flagellum continues to beat, and its movement may prevent stagnation of the medium surrounding the parasite in the vector. Its role in the free-swimming trypanosome is less clear. One likely possibility is that it circulates the contents of the flagellar pocket (Vickerman and Preston 1976). The pocket is an important site of entry and exit of materials in bulk (Fig. 1), as the pellicular microtubules preclude these activities elsewhere on the flagellate's surface.

Pinocytosis of proteins by trypanosomes has been observed by investigators using ferritin and other electron-dense tracers that can be located by electron microscopy. Details of the relationships between pinocytosis vesicles and lysosomes containing digestive enzymes (recognized by their positive reaction in Gomori staining for acid phosphatase) have been elucidated by Langreth and Balber (1975). Ferritin is taken up in spiny vesicles (Fig. 1) pinched off from the flagellar pocket

lining. These vesicles fuse with an elaborate network of smooth-membraned cisternae that appear to store the protein. Primary lysosomes from the Golgi complex fuse with parts of this network presumably digesting its contents. In some stages of the life cycles of certain trypanosomes, e.g., the intracellular amastigote and culture epimastigote forms of *T. cruzi*, but never Salivaria, a specialized cytostome and cytopharynx are present to mediate such "heterophagy." The protein sequestering system of *T. brucei* is so well developed that the uptake of host serum protein might be envisaged as taking place on a massive scale in relation to the size of the organism. Serum albumin has long been recognized as a prolonger of the active life of bloodstream trypanosomes in vitro and might be expected to be consumed avidly in vivo. Quantitative estimates of its uptake, however, have proved surprisingly low — 700 ng/h/mg trypanosome protein for monomorphic (slender) bloodstream *T. rhodesiense* though this figure was double for a pleomorphic line (A.H. Fairlamb personal communication), implying greater activity on the part of the stumpy trypanosomes.

Autophagy — the digestion of isolated islands of cytoplasm containing unwanted organelles — also appears to be more active in the stumpy forms (Langreth and Balber 1975). The cytoplasm to be destroyed is first walled off by membrane (probably supplied by the adjacent Golgi complex) to form an autophagic vacuole (Fig. 2) with which primary lysosomes fuse. Amino acids released from autophagic vacuoles may be utilized as an energy source in the stumpy form, which is activating its proline oxidase system.

The undigested remains of heterophagic and autophagic vacuoles are cast out into the flagellar pocket along with their accompanying lysosomal enzymes (Fig. 1, 2, and 3). This exocytosis of residual bodies may be of considerable significance in relation to pathogenesis. Trypanosome lysosomes contain acid proteases, leucine aminopeptidase, phosphatases, phosphodiesterases, deoxyribonucleases, α-mannosidase, β-hexosaminidase, and lipase (Venkatesan, Bird, and Ormerod 1977; Steiger 1975) as well as phospholipases (Tizard et al. 1977). Their destructive effects have not been fully investigated, but Tizard et al.p.103 have explored phospholipase and its role in the generation of lytic fatty acids.

The residual bodies exocytosed by *T. brucei* may be quite large in relation to the size of the trypanosome (Fig. 3). They may embody a mechanism for release of common (i.e., nonvari-

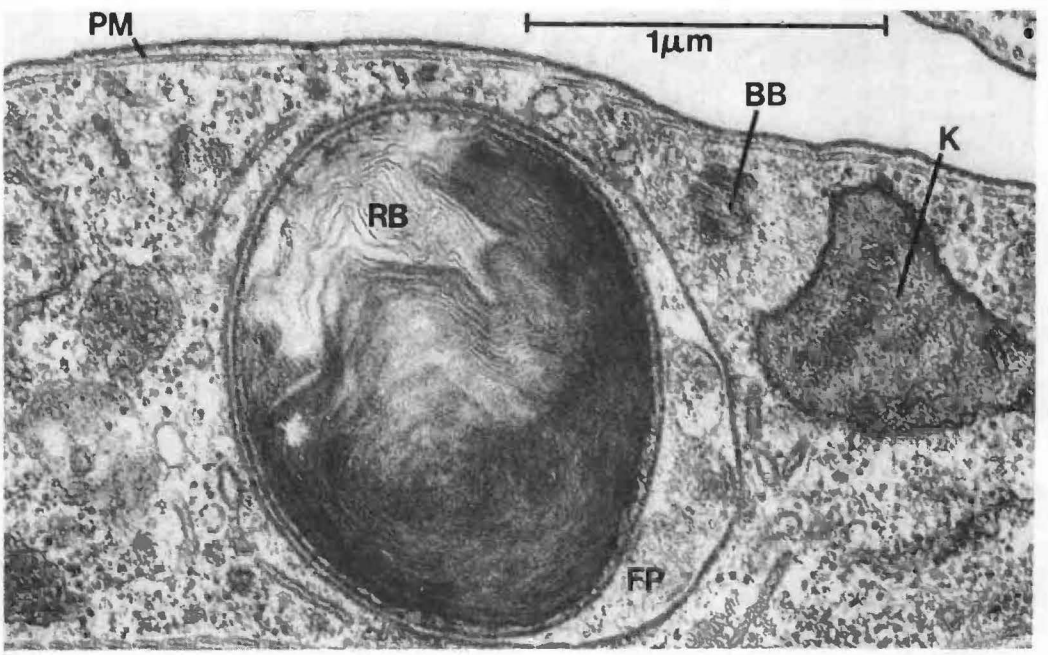

Fig. 3. *Electron micrograph of longitudinal section of the distended flagellar pocket (FP) of* T. brucei *(bloodstream stumpy form) containing large residual body (RB), which has the structure of a liposome, i.e., several concentric membrane layers. Surface coat on the membrane lining the pocket and covering the residual body as well as the trypanosome surface increases the apparent thickness of the membrane at this low resolution. Other labeled structures are the basal body (BB), pellicular microtubules (PM), and the kinetoplast (K).*

able) antigens before the wholesale liberation of these agents, which occurs when trypanosomes are destroyed by lytic antibody at a crisis. Discharge of these bodies as thick streamers from the flagellar canal has been described by Ellis, Ormerod, and Lumsden (1976).

The functional significance of many trypanosome cytoplasmic inclusions still eludes us. Trypanosomes have no means of storing energy as carbohydrate. These flagellates sequester triglyceride (Dixon, Ginger, and Williamson 1971), but it seems quite probable that, in the absence of an exogenous supply of respirable substrate, protein (of either internal or external origin) provides the only source of energy. Our recent X-ray microanalysis studies (Vickerman and Tetley 1977) of dense inclusions that lie to one side of a thick-membraned vacuole (Fig. 2 and 3) in trypanosome cytoplasm suggest that these are polyphosphate, but their role in trypanosome physiology is uncertain. Although they may function as phosphagens, i.e., to store phosphate bond energy when ATP production exceeds demand, they probably serve as phosphate reserves. The calcium and zinc associated with these granules suggest that they play a part in regulation of divalent cation concentrations in the cytoplasm — a function that can be vitally important in unicellular organisms. The mitochondrial cycle of the trypanosomes may be related to sequestration and release of divalent cations, as the ability of mitochondria to perform this function is well known. The pathological significance of polyphosphates discharged from the trypanosome on lysis deserves investigation, as these polyanions are utilized commercially as detergents.

One cytoplasmic organelle whose function has recently come to light is the glycosome (previously referred to as a microbody or peroxisome-like organelle by Vickerman and Preston 1976). Opperdoes and Borst (1978) have shown that in bloodstream *T. brucei*, it houses enzymes for the breakdown of glucose to glycerol-3-phosphate (G-3-P) and 3-phospho-glycerate (3-PG), which pass out into the cytoplasm. G-3-P is oxidized by molecular oxygen in the outer membrane of the mitochondrion and the dihydroxyacetone phosphate produced is shipped back to the glycosome for recycling. The 3-PG is converted to pyruvate in the cytoplasmic matrix, with linked phosphorylation of ADP, and the pyruvate is excreted by the trypanosome. The contribution of excreted pyruvate to pathogenesis is discussed in Newton p.17.

One of the most important trypanosome components in any discussion of pathogenesis is the surface membrane — the interface between parasite and host. In bloodstream African trypanosomes, the plasma membrane proper is overlaid by a surface coat of glycoprotein, 12–15 nm thick as seen in electron micrographs of sections (Vickerman 1969). Compact and dense in *T. brucei* and *T. congolense*, this coat is somewhat diffuse in *T. vivax*. An even more diffuse coat is present in the stercorarian trypanosomes such as *T. lewisi* and *T. cruzi* bloodstream forms. The coat appears to be an adaptation to life in the mammalian host, for it is discarded in the vector until it is reacquired at the metacyclic stage of development. In the Salivaria, it contains the variable antigen (VA) of the trypanosomes (Vickerman 1969b; Vickerman and Luckins 1969; Cross 1975; Fruit et al. 1977), and antigenic variation occurs when one coat is replaced with another of different antigenic type (reviewed by Cross 1978a; Vickerman 1978). The way in which replacement is instigated eludes us, but we believe that the change is phenotypic (i.e., genetic mutation is not required), that it occurs in only a small number of individuals (heterotypes) in a given population (approximately 1 in 10 000), and that antibody is not necessary for induction of the switch to expressing a new VA glycoprotein.

Cytochemical, especially lectin-binding, techniques indicate that in *T. brucei* the carbohydrate moieties attached to the single polypeptide chain of the variable antigen glycoprotein are located close to the membrane proper (Wright and Hales 1970; Steiger 1975; Cross and Johnson 1976; Seed, Seed, and Brindley 1976; Renwrantz and Schottelius 1977) and may even play a part in attachment of the molecule to the membrane. Whether in all variable antigen types of the subgenus Trypanozoon it is unexposed and plays no part in host–parasite interactions remains controversial (Baltz, Baltz, and Pautrizel 1977). In *T. congolense* (Rovis, Barbet, and Williams 1978; Jackson, Honigberg, and Holt 1978), *T. vivax* (Tetley unpublished observations), and the stercorarian trypanosomes (Dwyer 1976; Dwyer and D'Alesandro 1976), carbohydrate groups appear to be exposed on the surface coat rather than concealed.

The ease with which variable antigen is dissociated from the surface of the trypanosome in vitro suggests that it is a peripheral protein, i.e., has weak attachment to the membrane. In addition, the VA molecules appear to be mobile over the surface of the trypanosome in that they can be capped by homologous antibody in the indirect immunofluorescence reaction (Barry in press) and are lost uniformly from the surface as shown by electron microscopy and immunofluorescence studies on the trypanosomes transforming to culture forms in vitro (Barry and Vickerman in press). The

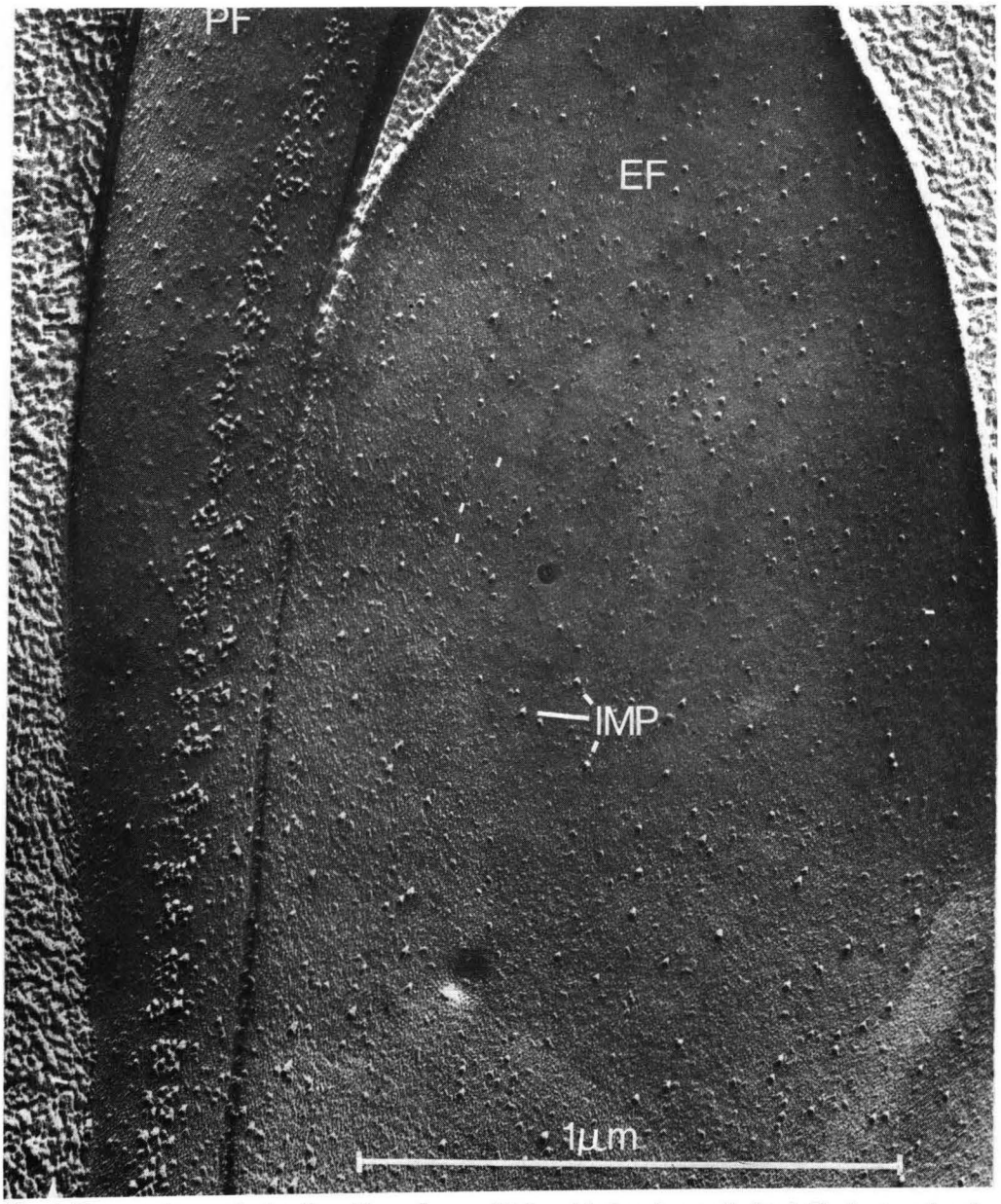

Fig. 4. *Electron micrograph of replica of freeze-fractured* T. brucei *(cultured procyclic form). The fracture plane has passed along the centre of the plasma membrane of the body (right) and the attached flagellum (left), revealing intramembranous particles (IMPs, integral proteins or lipoprotein complexes) that are embedded in the lipid bilayer. The extracellular face (EF) of the body membrane is shown and the protoplasmic face (PF) of the flagellar membrane. The clustering IMPs (a band running the length of the PF) represent the desmosome-like attachments of the flagellum to the body. Arrow indicates direction of shadowing of the replica.*

strong antigenic character of the VA may be due to its release from the trypanosome either on its entire surface, perhaps after combination with antibody, or on those enigmatic structures the plasmanemes or ''filopodium-like appendages'' (Wright, Lumsden, and Hales 1970; Vickerman and Luckins 1969). These fine extensions of the body and flagellum (Fig. 1) are readily observed on trypano-

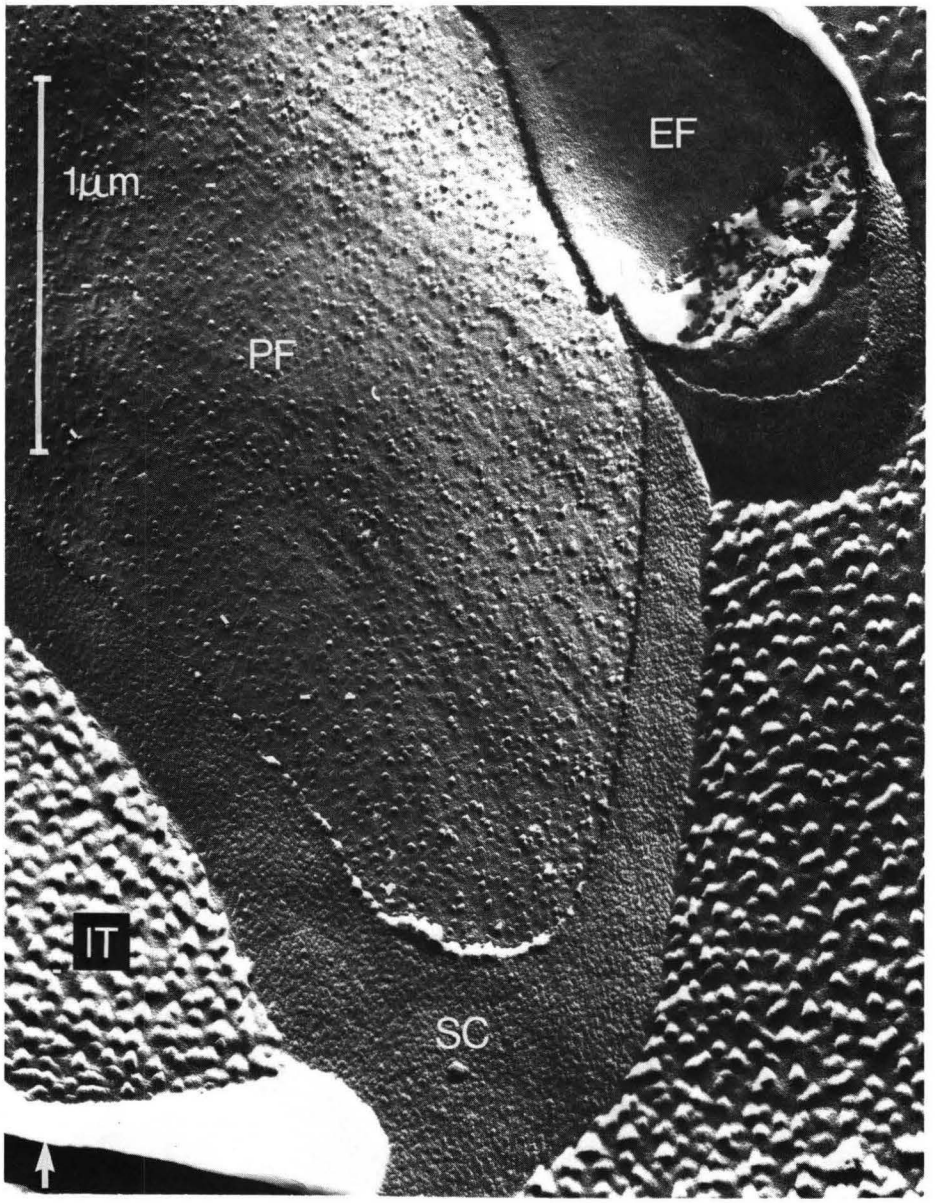

Fig. 5. *Electron micrograph of a replica of a freeze-fractured* T. vivax *(bloodstream form), which has been subjected to etching before shadowing. The protoplasmic face (PF) of the trypanosome body and the extracellular face (EF) of the flagellum plasma membrane are visible with their scattered intramembranous particles. In the etching process some of the covering ice is sublimed away to disclose the true surface of the membrane adjacent to the internal faces exposed by fracturing. The rough surface coat (SC) is visible because of the retreat of the ice table (IT).*

somes in vitro by light and electron microscopy, but definite proof of their formation in circulating blood is still lacking. Plasmanemes themselves are highly immunogenic (Herbert and MacAdam 1971), and their attachment to endothelial or red blood cells would provide a major source of antigen–antibody complexes to account for vascular damage.

Organized surface glycoproteins on flagellates are usually secreted by an active rough endoplasmic

reticulum-Golgi complex system, with externalization of new surface membrane bearing the secretion close to the flagellar bases. The existence of such a well developed secretory apparatus between the nucleus and flagellar pocket (Fig. 1 and 2) strongly suggests that a similar pathway exists in trypanosomes (Vickerman 1969). Owing to presence of the pellicular corselet of microtubules, it is difficult to see where new coated membrane could be inserted into the surface other than in the flagellar pocket. The possibility that secretion is controlled by lysosomal enzymes (as in the coccolithophorid flagellates)(Pienaar 1971) also exists. Secretion of a mitogen by salivarian trypanosomes has been suggested (Esuruoso 1976; Mansfield, Craig, and Stelzer 1976) and that variant antigen itself has a mitogenic effect has been postulated.

There is now convincing evidence that the soluble "exo-antigen" believed by Weitz (1960) to be a secretion is surface coat material liberated from the trypanosomes into the surrounding medium (Allsopp, Njogu, and Humphreys 1971). Whether the material is also liberated in vivo from viable trypanosomes is a matter of considerable interest to pathologists who are investigating complement activation by antigen–antibody complexes and its role in inflammatory disease (see Boreham p.114). Musoke and Barbet (1977) found that purified (soluble) variable antigen of *T. brucei* activated complement via the classical pathway, but a particulate fraction from the same clone of trypanosomes activated the alternate pathway of complement fixation. This fraction probably included the surface membrane proper of the trypanosome. Uncoated trypanosomes, as represented by culture forms, are susceptible to lysis by normal serum. In *T. cruzi* lysis appears to be due to the activation of complement via the alternate pathway (Nogueira, Bianco, and Cohn 1975). It seems likely that the trypanosome membrane proper has complement-activating components that in the intact flagellate are inaccessible owing to the barrier presented by the surface coat (see Nielsen, Tizard, and Sheppard p.94).

In the short term, the glycoprotein coat protects vulnerable membrane proteins (such as substrate transport sites) from complement components and in the long term the coat provides a replaceable surface enabling the trypanosome to evade the host's immune response. But that the variable antigens are the only proteins exposed on the surface of the bloodstream trypanosomes is unlikely. De Souza and colleagues (1978) have demonstrated differentiated regions on the surface of *T. cruzi* using cytochemical staining at the electron microscope level and the freeze-fracture replica technique. Differentiated regions of the surface of salivarian trypanosomes may prove to be discernible using similar methods.

In the freeze-fracture technique, frozen cells are cleaved; their membranes tend to split along the middle of the lipid bilayer, the fracture plane passing over and under integral proteins. The proteins then stand out as "intramembranous particles" (IMPs) when metal-shadowed replicas are made of the exposed fracture faces (Fig. 4 and 5). The true surface of the membrane — for example the surface coat of bloodstream trypanosomes — can be revealed by freeze-etching, i.e, subliming away surface ice adjacent to the fractured region of membrane (Fig. 5). No regular packing of coat glycoprotein units has been observed. The density of intramembranous particles visible (summing counts made from both faces of the split membrane) is $\sim 3 \times 10^3/\mu m^2$ for bloodstream *T. brucei* (Vickerman and Tetley 1977), whereas the density of coat glycoprotein molecules was calculated by Cross (1975) to be $\sim 10^5/\mu m^2$ so that coat molecules outnumber IMPs 20 to 1.

The proteins (or lipoprotein complexes) represented by IMPs are probably heterogeneous in function as well as size. Most noticeable of the IMPs are those that lie in clusters along the flagellum-body junction (Fig. 4) the clusters presumably corresponding with the desmosomal attachments of the flagellum (for further details see Smith et al. 1974; Hogan and Patton 1976; Vickerman and Tetley 1977) and the particles with the intramembranous portions of molecules that bridge the gap, i.e., protrude beyond the surface coat. There may be similar membrane differentiations, for example, where *T. congolense* attaches to host endothelium or where non VAT-specific cytophilic antibody binds the trypanosome to a macrophage. We are confident that in the not too distant future the combination of freeze-etching with techniques for the labeling of specific membrane components will result in significant information on the organization of the trypanosome surface and, indirectly, lead to a deeper understanding of the intricate relationship between parasite and host.

Acknowledgments

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Biochemistry of variant antigens

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Abstract. At present, the genetic basis of the trypanosome's antigenic variation is unknown, but within the next 2 years, amino acid sequence studies and recombinant DNA techniques should provide many of the answers. Meanwhile, there have been some noteworthy findings on the structure and cross-reactivity of variant antigens. Available information indicates that the surface coat of a *T. brucei* cell is composed of 7 million molecules of a single glycoprotein forming a closely packed monolayer. The surface coat of other trypanosome species may be markedly different: to date only *T. congolense*, *T. evansi*, and *T. equiperdum* have been examined. The surface of the cell is the point of contact with the host and may play an important part in the pathogenesis of trypanosomiasis, the salient characteristic of which is antigenic variation. The antigenic specificity exhibited by the trypanosomes seems to derive mainly from the diversity of the amino acid sequence, although there may be common sequences among variant antigens. Until recently, similarities among the antigens have been obscured by their apparent uniqueness; however, Barbet et al., p. 38, have discovered remarkable cross-reactivity. The significance of such findings has yet to be realized.

In reviewing the biochemistry of variant antigens, it is difficult but desirable to avoid the twin pitfalls of superficial speculation and premature dogmatism. To avoid compounding any previous errors in these directions, I refer the interested reader to my other short reviews (Cross 1977 a,b, 1978 a,b). There is little new that can be said with certainty. However, I am encouraged by the current surge of work, which — I am confident — will shortly answer the question of the genetic basis of antigenic variation. I will briefly summarize our present knowledge and highlight the likely routes of further advancement. Some recent developments relating to immunologic cross-reactivity will also be described.

Structure of *T. brucei*

Only a few variant antigens from one serodeme of *T. brucei* have been characterized in detailed biochemical terms, and the results may not be representative for all isolates of *T. brucei*. In fact, some aspects — for example, the location of carbohydrate side chains on the polypeptide chain and the reaction of intact cells with lectins — may differ from one variant to another.

The surface coat of each antigenically distinct and homogeneous *T. brucei* population appears to be composed of a single characteristic species of glycoprotein. The purified variant glycoproteins contain a single polypeptide chain of 65000 molecular weight (tested on sodium dodecyl sulfate polyacrylamide gels). The area of the trypanosome cell surface is similar to that of an erythrocyte (Cross 1975) and may be covered entirely by the glycoprotein, which at 65000 molecular weight constitutes about 7–10% of the total cell protein ($7-10 \times 10^6$ molecules of glycoprotein/cell) and could be a closely packed monolayer. At least, this idea forms the simplest working hypothesis as long as there is no conflicting evidence. How the variant glycoproteins are attached to the trypanosome surface is unknown. Although they appear to be loosely bound, they may be released by proteolytic cleavage of a larger molecule that penetrates the lipid bilayer. This possibility is encouraged by the apparent susceptibility of the carboxy terminus to proteolytic degradation (Johnson and Cross 1979) and the marked heterogeneity of variant glycoprotein antigens found by other workers (see references in Cross 1975), the latter probably largely attributable to proteolytic degradation.

Variant glycoproteins isolated from *T. brucei* have characteristic isoelectric points, amino acid, and carbohydrate compositions (Cross 1975, 1977 a,b; Johnson and Cross 1977). Glycoproteins

isolated so far from about 15 variants of several cloned isolates of *T. brucei* all have some unique characteristic, even in such a crude criterion as amino acid composition. Such distinctiveness may disappear as a wider range of antigens is biochemically characterized. Reisner and Westwood (1977) used our amino acid composition data and attempted to derive estimates of secondary and tertiary structural parameters, but it is debatable whether the methods they used can reveal any information of real value.

Antigenic specificity of the variant glycoproteins appears to be primarily due to extensive diversity in the amino acid sequence. Sequence variation occurs throughout the polypeptide chain, but whether it is as extensive as is found at the amino terminus (Fig. 1) remains to be seen. Amino acid sequence studies are under way in at least two laboratories to determine the full extent of variability and to illuminate the underlying genetic mechanisms involved in the generation of diversity. In the 2 years since Bridgen, Cross, and Bridgen (1976) obtained the first N-terminal sequence data, however, amino acid sequencing has almost been made obsolete by DNA sequencing and in vitro recombinant techniques. If there are no further restrictions on recombinant DNA work, it seems likely that amino acid sequencing will not be productive after 1980. At present, amino acid sequencing has the advantage of being immediately applicable to the problem without using isolation laboratory facilities.

One factor that gives cause for optimism concerning the results from gene mapping is that, relative to mammalian DNA, the nuclear DNA of trypanosomes seems simple (Hoeijmakers and Borst communication at the 15th trypanosomiasis seminar of the Overseas Development Ministry, Edinburgh 1978). In fact, the nuclear DNA of *Leishmania tarentolae* has a complexity only three times that of the bacterium *Escherichia coli* (Wesley and Simpson 1973).

A desirable initial step in gene cloning is the partial purification of variant antigen messenger RNAs. This has already been reported by two laboratories (Eggitt, Tappenden, and Brown 1977; Williams et al. 1978). Williams and coworkers' results suggest that the abundance of some RNA species depends on the variant but that all RNA species are present in the different variants.

It does not seem worthwhile to speculate on the genetic basis for the generation of antigenic diversity. Sufficient experimental evidence should be available within 18 months to answer the question of whether a variant antigen is coded by one structural gene or by some mechanism splicing several genetic elements. The answers may hold some surprises but will probably not be more startling and exciting than the recent revelations of gene organization in viruses and eukaryotic cells. The classical dogma that one gene codes one protein has been superseded by the discoveries that one gene may code as many as three proteins in certain viral genomes, that several genetic segments may code one immunoglobulin polypeptide, and that intervening nontranslated sequences (Introns) occur throughout eukaryotic structural genes and necessitate RNA splicing to form the translated messenger. Any of these could be involved in the encoding of variant antigens.

The carbohydrate differs among variant antigens in amount (7–17% by weight), composition, and location on the polypeptide backbone, all of which may accentuate antigenic heterogeneity. Carbohydrate is generally associated with secreted polypeptides, but I suggest that a small element of

Variant	10																20
99	thr.	ans.	asn.	his.	gly.	leu.	lys.	leu.	gln.	lys.	ala.	glu.	ala.	ile.	cys.	lys.	met. cys. lys. glu.
49	ala.	lys.	glu.	ala.	leu.	glu.	tyr.	lys.	thr.	trp.	thr.	asn.	his.	cys.	gly.	leu.	ala. ala. thr. leu.
48	thr.	asp.	lys.	gly.	ala.	ile.	lys.	phe.	glu.	thr.	trp.	glu.	pro.	leu.	gln.	leu.	leu. thr. gln. asp.
55	ala.	glu.	ala.	lys.	ser.	asp.	thr.	ala.	ser.	gly.	ser.	val.	asn.	ser.	pro.	gln.	thr. glu. ala. *thr.*
221	ala.	ala.	glu.	lys.	gly.	phe.	lys.	gln.	ala.	phe.	trp.	gln.	pro.	leu.	cys.	gln.	val. ser. glu. glu.

	30												
	arg.	lys.	val.	ala.	thr.	gly.	val.	leu.	thr.	lys.	leu.	lys.	*ser.* his. ile.
	phe.	gly.	asn.	leu.	tyr.	asn.	lys.	ala.	lys.	.	.	asn.	leu. asp.
	tyr.	.	*ala.*	gln.	leu.	ala.	lys.	thr.	leu.	gln.	*arg.*	ala.	leu. asp.
	leu.	asp.	asx.	glx.	pro.	*Lys.*	*gly.*	*ala.*	leu.	phe.	thr.	leu.	glx. *ala. ala. (ala could be ser)*

Fig. 1. *N-terminal amino acid sequence of five variant antigens from* T. brucei *427. Residues in italics are uncertain assignments (from Bridgen, Cross, and Bridgen 1976 and Johnson and Cross 1979).*

essential carbohydrate in the variant glycoproteins contributes to secretion. Due to the diversity in the amino acid sequence, however, it may be that some sites along the polypeptide are fortuitously glycosylated simply because they possess the sequence recognized by the glycosylating mechanisms.

Antigens of Other Salivarian Trypanosomes

My own preliminary experiments with a dis-kinetoplastic clone of *T. evansi* SAK showed very different results from those using *T. brucei* (Cross 1977a). Purified glycoprotein tested on SDS polyacrylamide gels under nonreducing conditions showed a single component of 96 000 molecular weight. Reducing conditions yielded a major band at either 65 000 or 45 000 molecular weight, depending on the fraction separated by isoelectric focusing (Fig. 2). The reduced glycoprotein was closer to that found in *T. brucei*, but the results introduced new questions that were outside the scope of my current investigations.

Identification and characterization of the surface coat components of *T. congolense* has been reported by two laboratories. Using lactoperoxidase-catalyzed ^{125}I iodination or galactose-oxidase oxidation followed by NaH_3B_4 reduction, Rovis, Barbet, and Williams (1978) identified a 56 000 molecular-weight glycoprotein in what appeared to be a single polypeptide. The glycoprotein was prepared from a trypanosome clone that was homogeneous according to immunofluorescence criteria. It showed three components differing in isoelectric point. The authors postulated that the heterogeneity resulted from slight proteolytic degradation of the antigen. Only 20% of the surface glycoprotein of *T. congolense* was soluble in the absence of detergent, contrasting with 80% for *T. brucei* (Cross 1975) but correlating with the findings (Cross 1977b and unpublished data) for *T. evansi* surface glycoprotein (10–20%). The question is whether the soluble antigen is truly the entire antigen.

Reinwald, Risse, and Selker (in press) used ^{35}S-labeled diazotized sulfanilic acid to identify the major surface component of *T. congolense*. Most of the label was recovered in a 57 000 molecular weight component, with a small amount in a secondary component of 50 000 molecular weight. Additional studies are needed to show whether the results are typical of the species.

I feel it would be valuable to compare the structural chemistry of *T. brucei* and *T. congolense* antigens and to determine whether different trypanosome species have variant antigen specificities in common. Such evidence would cast light on trypanosome evolution.

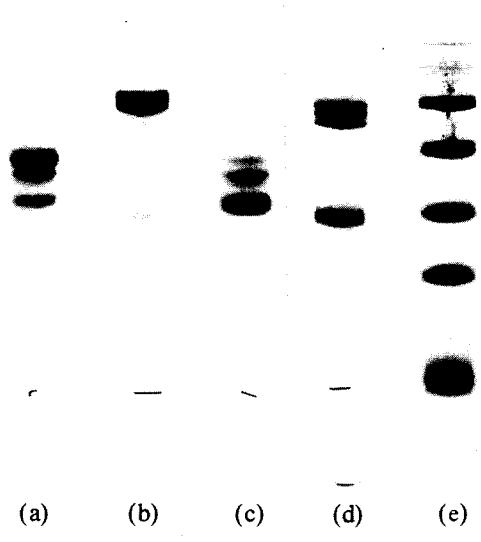

Fig. 2. *Surface glycoproteins of* T. evansi *SAK clone 65. Cells were labeled and fractionated as described for* T. brucei *(Cross 1975). The electrofocusing column contained several glycoprotein peaks. Two areas consisting of tubes 112/123 and 124/135 were pooled and examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular weights of five marker proteins tested on parallel gels were 96 000, 68 000, 45 000, 29 000, and 13 000; (a) fraction 112/123 reduced, (b) nonreduced, (c) fraction 124/135 reduced, (d) nonreduced, and (e) the standards.*

Baltz, Baltz, and Pautrizel (1976) isolated glycoprotein antigens from *T. equiperdum* using affinity chromatography of soluble extracts on Concanavalin-A-sepharose. The molecular weight of the single glycoprotein band obtained from two clones was the same as that reported for *T. brucei* (65 000), but the glycoprotein from a third clone appeared to exist as a dimer when gel electrophoresis was done under nonreducing conditions. A portion of the glycoprotein could not be eluted from the affinity column unless 0.2% Triton detergent was included in the eluting fluid. Preliminary analysis showed that each clone-specific glycoprotein fraction contained 4–7 components of different isoelectric points. The authors attributed this "microheterogeneity" to ". . .minor modifications in the (polypeptide) interior of each molecule." I feel that minor proteolytic degradation is a more likely explanation. Amino acid analyses and N-terminal sequencing of several *T. equiperdum* variant glycoproteins showed a degree of variability similar to that found in *T.*

brucei, but sugar analyses seemed to indicate the presence of a constant carbohydrate chain (Baltz et al. 1977).

To date, no characterization of the surface antigens of *T. vivax* has been reported, and there has been no advance in understanding the possible role of absorbed serum proteins in the surface coat of *T. vivax*.

In all species of trypanosomes, there is a paucity of studies on the makeup of the plasma membrane, although Martin, Voorheis, and Kennedy (1978) recently reported isolating plasma membranes from *T. brucei* and characterizing a membrane-bound adenyl cyclase. This work may provide the impetus for further studies.

Control of Antigenic Variation

The molecular and genetic mechanisms involved in the control of antigenic variation in bloodstream trypanosomes may now be studied in vitro. A long-term culture has recently become available (Hirumi, Doyle, and Hirumi 1977; Hill et al. 1978) and has already been used for baseline studies on antigenic stability (Doyle et al. submitted for publication). Before the advent of long-term culture in vitro, other workers tried to study antigen switching using short-term cultures (Taylor and Cross 1977). Although they were able to show synthesis and secretion of surface antigen, they were unable to observe antigen switching. This work is mentioned only because short-term cultures could still be used for in vitro studies in which cell replication is not required.

In addition to the question of how antigenic variation is programed within the mammalian host are the questions of what mechanisms cause antigen synthesis to be switched off in the midgut of *Glossina* (or in culture at 25 °C) and turned on again in the salivary glands. Controversy also currently revolves around the antigenic homogeneity or heterogeneity of metacyclic trypanosomes (Vickerman 1978; Le Ray, Barry, and Vickerman 1978; see also Jenni p.49 and Barry and Hajduk p.51).

Surface Coat in Pathogenicity

As the interface between a trypanosome and its host, the surface coat might be expected to play a major role in pathogenicity. For example, it may be related to the selective infectivity of the trypanosomes. Although Rifkin (1978) has recently demonstrated that high-density lipoprotein in human serum is responsible for the specific lysis of *T. brucei brucei*, her observation does not entirely account for the selective infectivity of certain trypanosomes to humans. How are Dr Rifkin's results to be reconciled with earlier claims that individual antigenic variants of *T. brucei* can exist in serum-sensitive and serum-insensitive forms (Van Meirvenne, Magnus, and Janssens 1976)? Does high-density lipoprotein have to breach the surface coat barrier (Cross and Johnson 1976) to exert its trypanocidal effect? If so, might the primary cause of serum sensitivity reside in differences in the stability of the plasma membrane linkage of variant glycoproteins?

The surface coat might also be expected to play an important part in tissue tropisms and immunopathology. Some possible implications of the ability of variant antigens to activate complement by the classical pathway have been discussed by Musoke and Barbet (1977).

Structure and Immunochemistry

Variant glycoproteins are susceptible to proteolytic cleavage, and this property has been successfully used to distinguish functional domains of immunoglobulins and to isolate large, soluble fragments of membrane-penetrating insoluble antigens of mammalian cell surfaces. Johnson and Cross (1979) used tryptic cleavage of six variant glycoproteins from *T. brucei* to distinguish two conformationally distinct domains representing the amino and carboxy termini of the polypeptides. Within 5 minutes, native glycoproteins were cleaved; a large N-terminal fragment (48 000 – 55 000 molecular weight, depending on the variant) was apparent together with one or more smaller C-terminal fragments. After 30–60 minutes the large fragment broke down further in some variants to 40 000–52 000 molecular weight fragments that, for at least the next 16 hours, were resistant to trypsin cleavage. The characteristic products of trypsin cleavage are summarized in Fig. 3. In four investigations, the large fragments proved to be the N-termini of the intact antigens; the C-terminal domains did not appear to have a constant amino acid sequence. The distinction between the N-terminal and C-terminal domains, which dissociate without the use of denaturing conditions or sulfhydryl reagents, may be significant in the organization and function of the glycoprotein on the trypanosome surface.

Whether variant antigens are distinct structural genes evolving from gene duplication and mutation or are formed through the splicing of gene ele-

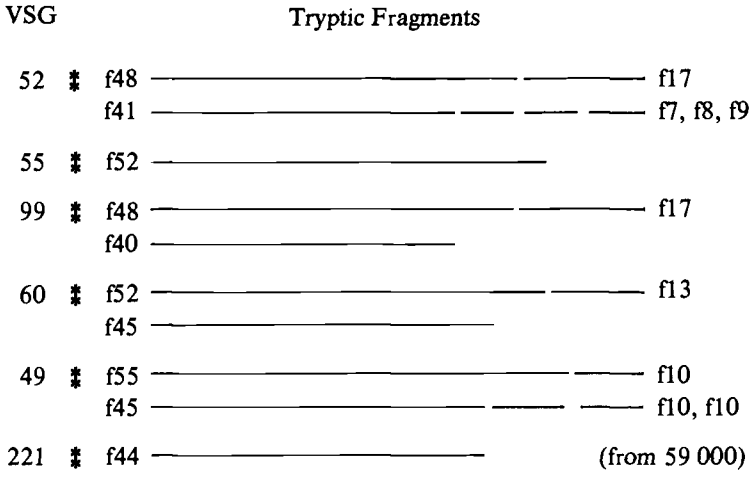

Fig. 3. Summary of the polypeptide fragments arising from tryptic cleavage of six native variant surface glycoproteins (VSG) (Johnson and Cross 1979). The symbols such as f48 refer to fragment 48 000 molecular weight, etc.; the asterisks indicate fragments that were sequenced at the N-termini. Variant 221 had an initial molecular weight of 59 000.

ments, they may very well have common amino acid sequences. There may also be resemblances that have not been apparent to date because of the limited numbers studied. However, the magnitude of the immunologic cross-reactions between variant antigens reported by Barbet and McGuire (1978) was quite unexpected, especially as it was found in a series of antigens I had investigated originally (Cross 1975).

Shortly before leaving Cambridge, in 1977, I had collaborated with F. van Knapen at the Rijks Institut voor de Volksgezondheid who performed a series of enzyme-linked immunoabsorbent assays using antisera against two purified *T. brucei* variant antigens. The results (Fig. 4) of the assays were puzzling because of the cross-reactions. Antigen 221 reacted strongly with heterologous antisera raised against purified 060 variant antigen and with antisera from rabbits infected with *T. brucei, T. rhodesiense, T. gambiense, T. vivax,* and *T. congolense*. It seemed unlikely that variant antigen 221 was being expressed in all these infections, but as the only other antigen included in the tests (060) did not show such widespread cross-reaction, the significance of the result was not apparent at the time.

Clearly, such strong cross-reactions could have significance for both the academic and practical considerations of antigenic variation. Dr Barbet will be presenting a paper on this topic, so I will limit myself to summarizing some observations I have made in attempting to confirm and extend his observations. The detailed results have been published elsewhere (Cross 1979 a, b).

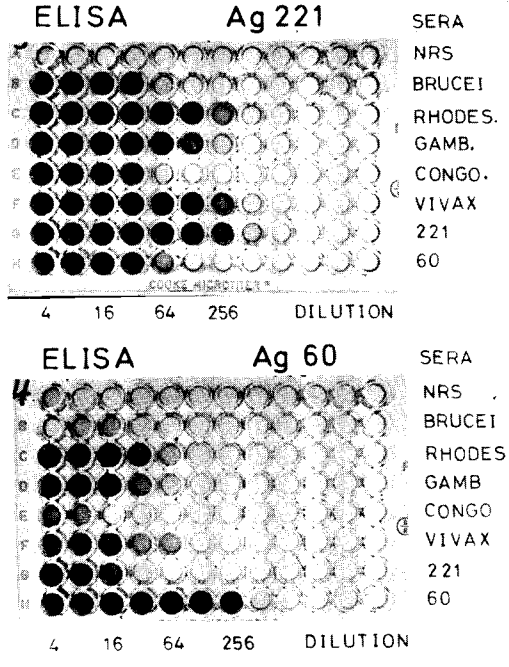

Fig. 4. Enzyme-linked immunoabsorbent (ELISA) assays of two purified variant glycoproteins, two antisera raised against purified glycoproteins, and four antisera arising from rabbits infected with T. brucei brucei *(Lugala 1066),* T. brucei gambiense *and* rhodesiense *isolated from humans,* T. congolense, *and* T. vivax. *NRS represents normal rabbit serum; each well contained 1 μg of purified antigen. Assays were performed by F. van Knapen, Rijks Institut voor de Volksgezondheid, Bilthoven, Netherlands.*

I found wide variations in the ability of seven heterologous variant antisera to precipitate four variant antigens, including three identical to those used by Barbet and McGuire. I exchanged antisera with them and found that my antisera had much lower or zero cross-reacting titres compared with theirs. Their antisera of four heterologous specificities were all of approximately equal and high efficiency in precipitating antigens 055 and 221 (50% precipitation of 10 ng antigen at serum dilutions of 10^2-10^3 fold) in our laboratory. When I adopted the immunization protocol used by Barbet and McGuire, the cross-reacting antisera titres only slightly increased. These findings suggest that variation between the responses of individual rabbits may be more important than the immunization protocol.

I have presented evidence (Cross 1979b) that the heterologous immunoprecipitation can be attributed to determinants in the C-terminal domain of variant antigens. N-terminal native tryptic cleavage fragments (Fig. 3) of molecular weights 52 000, 41 000, and 44 000 from variant antigens 055, 121, and 221 respectively were not precipitated by any heterologous antisera, and antisera raised against the N-terminal fragments of variant antigens 055 and 121 did not precipitate intact heterologous variant antigens. A glycopeptide fragment from the C-terminal domain of variant antigen 121 strongly inhibited the immunoprecipitation of antigen 055 by antiserum 121.

The C-termini of the native antigens appear to be very susceptible to proteolytic degradation, presumably because they are rather loosely folded. Thus, slight denaturation may expose common antigenic determinants that are normally masked by the native configuration of the polypeptide. Because native and denatured proteins have different antigenic specificities, it may be that some sequences in the polypeptide are conserved and that the cross-reactions have little relevance to the antigenicity of the native antigen in situ. Alternatively, cross-reactions may be attributable to common carbohydrate determinants (Barbet et al.p. 38). I did not entirely eliminate this possibility (Cross 1979a), and in fact in other studies with Johnson (1976, 1979), I noted that the carbohydrate is mainly near the C-termini of variant glycoproteins.

Cross-reacting determinants in trypanosome surface antigens

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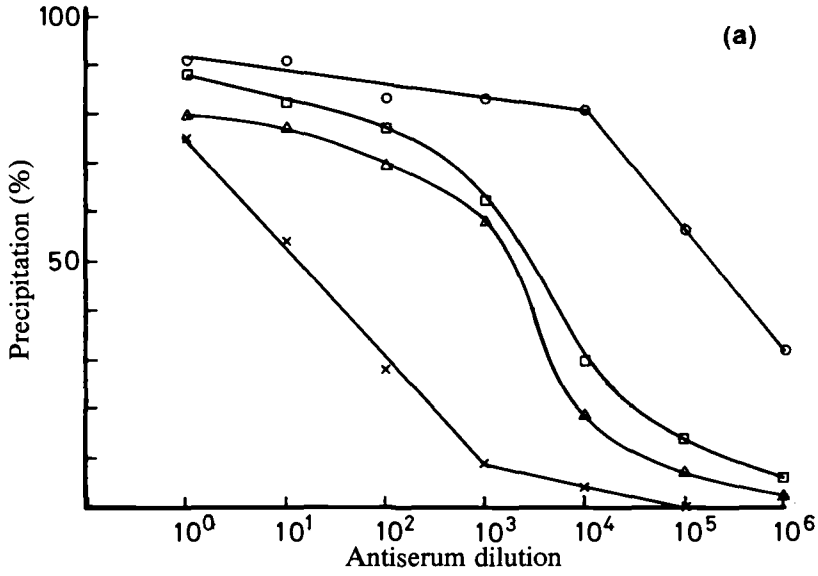
Abstract. We isolated surface glycoproteins from six clones of *T. brucei* and one of *T. congolense* and produced antisera against them. We then used radioimmunoassay techniques to test for cross-reactivity among the antigens. The tests followed a pattern: when homologous antiserum and radioactively labeled antigen were used, the unlabeled homologous antigen was the only effective inhibitor of precipitation; when heterologous antigen and antiserum were used, however, any of the variant antigens completely inhibited precipitation of the labeled antigens. These results indicate cross-reacting determinants in the trypanosome surface antigens, against which antibodies are produced by inoculation of the purified molecules. We then began to analyze the structure of the cross-reacting determinant. Comparing composite maps of antigens we found a few overlapping peptides, but the most encouraging results came from tests using periodate to cleave the carbon-carbon bonds in the variant antigen's carbohydrate component. This treatment abolished the antigen's ability to inhibit heterologous immunoprecipitation.

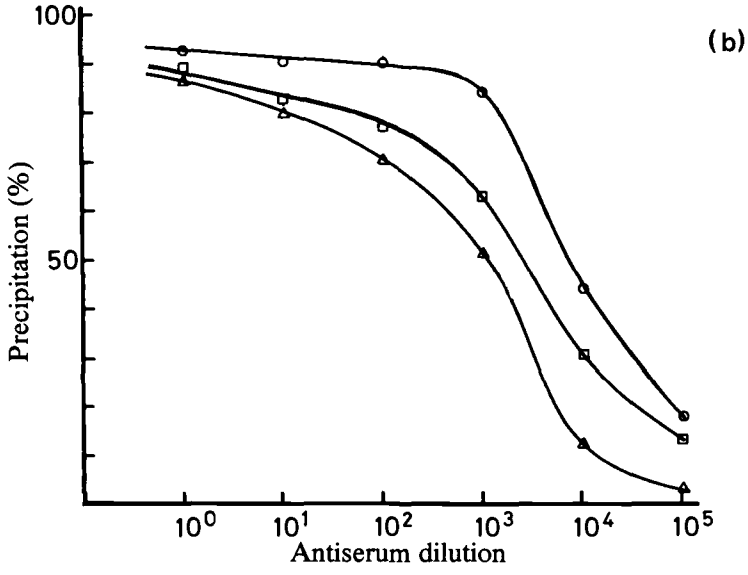
After G.A.M. Cross (1975) described a method for isolating and characterizing variable surface antigen glycoproteins from *T. brucei*, we at the International Laboratory for Research on Animal Diseases decided to use a radioimmunoassay method to investigate immunologic cross-reactions between the isolated antigens. Similar methods had been used successfully in studies on viral proteins (Strand and August 1975). Radioimmunoassay offered the advantages of great sensitivity and a minimal requirement for the amount of pure antigen, and used in combination with other biochemical structural methods, it promised a means to analyze degrees of relatedness among trypanosome variant antigens. At the beginning of the study, there was no evidence for biochemical or immunologic similarity between the isolated surface antigens, except for similarity in molecular weights (Cross 1975) and in susceptibility to limited tryptic cleavage (Bridgen, Cross, and Bridgen 1976).

We isolated surface glycoproteins from six clones of *T. brucei* and one of *T. congolense* (Rovis, Barbet, and Williams 1978). Three of the *T. brucei* glycoproteins, 049, 052, and 055, had been characterized previously (Cross 1975), providing us with reference data on the isoelectric points. We purified and characterized the other *T. brucei* glycoproteins, which were from a different original field isolate. Then we made antisera to each of the isolated glycoproteins, using rabbits (three injections, 10 μg protein each). We preferred this immunizing dose because of the low antigen cost, the adequacy of the antiserum produced, and the fear that high immunizing doses would be more likely to produce a detectable antibody response against any unobserved contaminants in the glycoprotein preparation. Each antigen was labeled with ^{125}I by the chloramine-T method (Hunter 1973) and a double antibody immunoprecipitation system devised where antigens were tested for binding by various dilutions of rabbit antisera. The rabbit antibodies were precipitated by a calculated amount of sheep anti-rabbit IgG serum.

Fig. 1 shows the results. ^{125}I-labeled 055 antigen was precipitated (Fig. 1a) by the homologous antiserum, anti-055, and three heterologous antisera anti-049, anti-052, and anti-*T. congolense* X4. Fig. 1 (b) shows the converse where a single antiserum, anti-052, precipitated three different labeled antigens — homologous ^{125}I-052 and two heterologous ^{125}I-049 and ^{125}I-055. In all cases, the homologous antiserum titrates out further, but all the heterologous antisera bind the antigens. SDS-



○ = ^{125}I-055 and anti-055 serum
△ = anti-049 serum
□ = anti-052 serum
× = anti-*T. congolense* X4 serum



○ = anti-052 serum and ^{125}I-052 VSSA
△ = ^{125}I-049 VSSA
□ = ^{125}I-055 VSSA

Fig. 1. *Double antibody radioimmunoassay plots for ^{125}I-labeled VSSAs and anti-VSSA sera.*

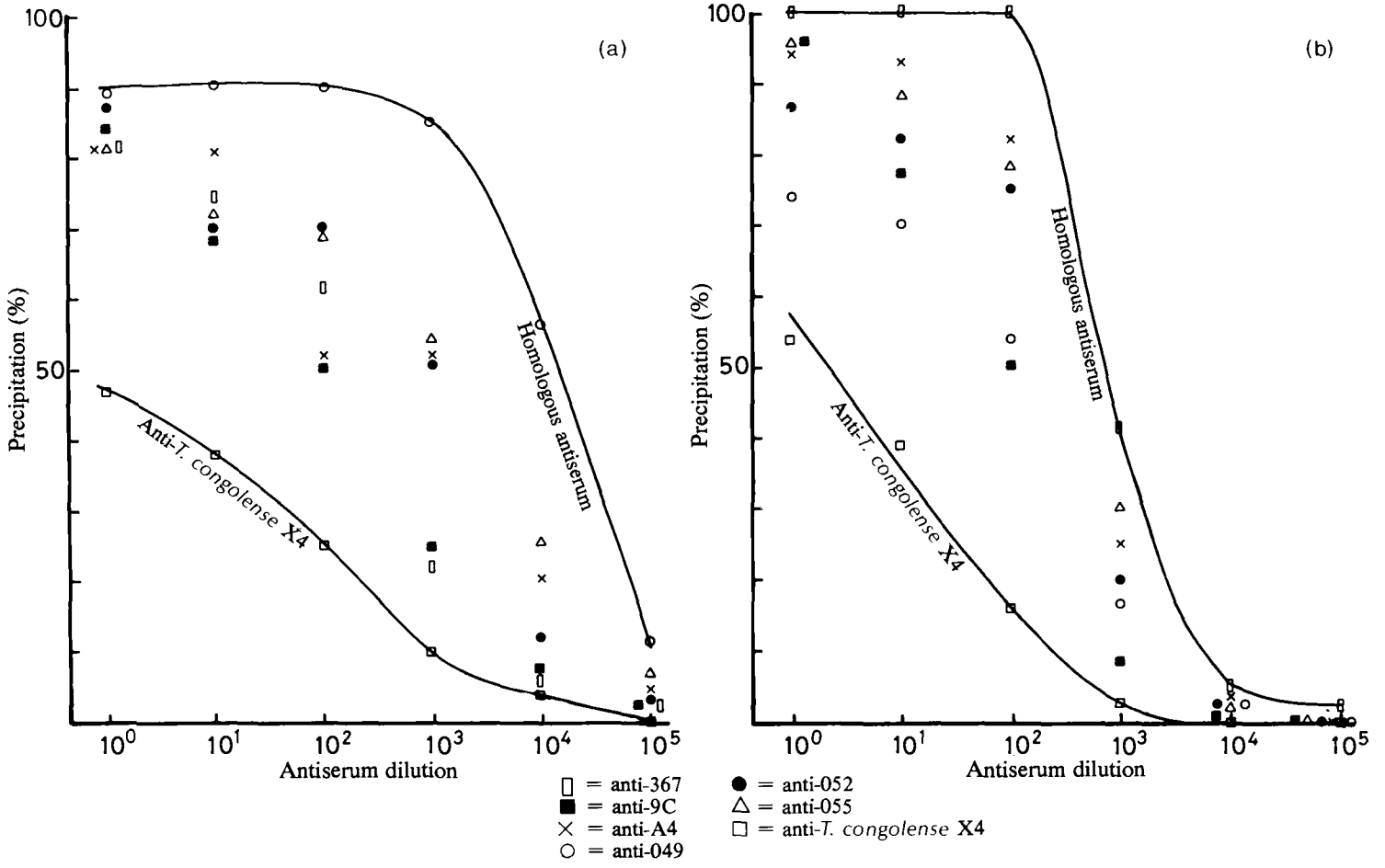


Fig. 2. *Double-antibody radioimmunoassay plots for ^{125}I-049 VSSA (a), ^{125}I-367 VSSA (b), and anti-VSSA sera.*

gel electrophoresis of the immunoprecipitates demonstrated that all radioactivity was in a peak of 65 000 apparent molecular weight, corresponding to that of the isolated antigen. Two different iodinated *T. brucei* surface antigens were precipitated with each antivariant antigen serum (Fig. 2).

In the next series of experiments, we investigated inhibition of precipitation of labeled antigen by unlabeled antigen. Various amounts of unlabeled antigens were added to the assay mixture; then the rabbit antiserum was added in a dilution that, in the absence of inhibitor, precipitated 50% of the labeled antigen.

This assay conformed to a general pattern (Barbet and McGuire 1978). When homologous antiserum and labeled antigen such as ^{125}I-055 × anti-055 were used, only the unlabeled homologous antigen, e.g., 055, was an effective inhibitor. However, when heterologous antigen and antiserum, such as ^{125}I-055 × anti-052 were used, any of the isolated variant antigens completely inhibited precipitation of the labeled antigens. The results, therefore, show cross-reacting determinants in the trypanosome surface antigens, against which antibodies are produced by inoculation of the isolated molecules. Antibodies raised against one antigen will bind to any other purified antigen and binding may be inhibited by any third purified surface antigen — a phenomenon suggesting that the cross-reacting determinant(s) is similar in all cases. As the homologous reactions are not inhibited by heterologous antigen, it is likely that antibodies to variable determinants predominate over those against cross-reacting determinants.

We were concerned to eliminate the possibility that cross-precipitation was due to artifactual contamination of the glycoprotein preparations, with either other variant antigen or another soluble antigen, which was not variant. We believe there are strong arguments against this possibility:

- Antigens that have widely differing isoelectric points cross-react very well and should, therefore, be separated by the isoelectric focusing step.
- Hypothetically, isoelectric focusing should also separate at least some of the glycoprotein antigens from an impurity containing cross-reacting determinants which would, presumably, have a single isoelectric point.
- By indirect immunofluorescence, we found that all antisera react specifically with a single clone of trypanosomes even using undiluted sera.
- SDS-gel electrophoresis of immunoprecipitates shows all radioactivity in a peak of 65 000 apparent molecular weight.
- It is possible to precipitate nearly all the radioactivity in an antigen by a heterologous antiserum; therefore, one is not dealing with a minor, contaminating impurity.

We have, during the last year, performed many different purification procedures, in addition to those described by G.A.M. Cross (1975), that end with isoelectrofocusing. These include, for example, gel filtration in different varieties of Ultragel, Sephadex, and Biogel columns; purification on ion exchange resins, and isoelectrofocusing in the presence of the nonionic detergent NP40. None of the additional procedures affects the ability of the variant glycoprotein to cross precipitate or to inhibit the heterologous cross-reactions. We, therefore, believe that the cross-reacting determinants are an integral part of the variant glycoprotein molecule.

Cross-reacting antibodies do not produce fluorescence of live trypanosomes, nor do they lyse or neutralize them. This finding signifies that the relevant determinants are not exposed when the variant glycoprotein is in a normal conformation on the surface of a trypanosome. The existence of cross-reacting determinants, however, cautions against using immunofluorescence of fixed trypanosomes to diagnose antigenic type, without confirmation from other tests such as neutralization or immune lysis.

Since these results, our work has diversified into two main areas. The first is a continuing study to try and define the structure of cross-reacting determinants; the second is to use some of the methods that were designed during the initial studies in other areas of trypanosome research.

An example of the latter is a project applying radioimmunoassay techniques to studies on the variant antigen content of the infective, bloodstream type of trypanosome growing in cell culture at 37 °C (Hirumi, Doyle, and Hirumi 1977). The major differences between such trypanosomes and those that have been cultured previously are their infectivity to the mammalian host and the presence of surface coat. In future, these cultures could be used to study the rate of synthesis and turnover of variant antigen and the factors that effect and control this. In the meantime, we wanted to establish whether these trypanosomes retain a fixed complement of variant antigen for long periods of time.

To answer this question, we used radioimmunoassay to quantify the relative amounts of variant antigen that could be released from trypanosomes of a specific antigenic type growing in culture for different lengths of time. We then

compared this figure to one obtained from trypanosomes of the same antigenic type growing in rats. These had never been in culture and had not been derived from trypanosomes that had been cultured.

We isolated variant antigen corresponding to the antigenic type grown in culture (221) and performed the homologous inhibition radioimmunoassay, ^{125}I-221 × anti-221 (precipitation inhibited by 221 in NP40), using unlabeled 221 glycoprotein as a calibration standard. We then used, as an unknown inhibitor, various dilutions of a supernatant fluid (230 000 g, 60 min, 4 °C) from known numbers of trypanosomes treated with detergent (0.5% NP40 and sonication for 2 minutes on ice). We ensured, by immunofluorescence, that greater than 99% of trypanosomes bore the correct variable antigen, and then we performed the assay. The results demonstrated no marked difference between the amount of 221 antigen released from bloodstream-grown trypanosomes and that from trypanosomes cultured at 37 °C (Table 1).

Table 1. Amounts of 221 antigen released from bloodstream-grown and cultured trypanosomes.

Trypanosome	Source	Time in culture	221 glycoprotein mg/10^{10} trypanosomes
Mouse-infective	Culture	1 mo	7.2
Mouse-infective	Culture	2 y	7.5
Mouse-infective	Bloodstream	n.a.	7.4
Noninfective[a]	Culture	–	<0.01

[a]These forms were derived by transformation of 221-infective culture forms at 25 °C.

n.a. = not applicable

Using supernatant fluid from 221 trypanosomes that had been transformed to noninfective forms at 25 °C, we observed no inhibition. The value, <0.01 mg/10^{10} trypanosomes, represented the lower detection limit using approximately 10^6 trypanosomes.

While performing these assays, we hoped to discover whether the cross-reacting determinants in

Table 2. Ability of 221 antigen to inhibit heterologous immunoprecipitation.

Reaction	Inhibiting antigen	Treatment	Antigen added (ng)	Inhibition (%)	Comments
Ag-221 (^{125}I) v anti-RMI	221	Control (incubated	1000	89	Antigen intact after
		16 h at 37 °C	100	42	treatment
		then inactivated			(shown by
		trypsin was	10	5	SDS-gel
		added, 1:10)			electrophoresis)
	221	Incubated 16 h	1000	71	Antigen
		at 37 °C with			extensively
		trypsin (1:10)	100	28	degraded
		then soybean			(shown by
		trypsin inhibitor	10	10	SDS-gel
		added			electrophoresis)
Ag-055 (^{125}I) v anti-221	221	Control	1000	98	Antigen binds to Concanavalin
			100	85	A and reacts with homologous
			10	10	antiserum by immunodiffusion
	221	Incubated 24 h	1000	0	Antigen does
		at 4 °C in the			not bind to
		dark with periodate			Concanavalin A
		(250 moles/mole	100	0	but reacts with
		glycoprotein);			homologous
		acetate buffer	10	0	antiserum by
		pH 4.75			immunodiffusion
	221	Incubated 46 h	1000	17	
		at 4 °C in the			
		dark with periodate			
		(60 moles/mole			
		glycoprotein);	100	7	
		acetate buffer			
		pH 4.75	10	0	

the variant antigen were present in the different types of cultures. This was achieved simply by using the same supernatant fluids to inhibit a heterologous immunoprecipitation reaction. The result was, in principle, the same as inhibition of the ^{125}I-221 × anti-221 reaction. That is, the supernatant fluid derived from the infective bloodstream forms cultured at 37 °C could inhibit the heterologous reaction to completion, whereas that derived from the noninfective forms cultured at 25 °C did not inhibit the reaction. Thus, on transformation, infective bloodstream type 221 trypanosomes lost all 221 antigen detected by the homologous inhibition radioimmunoassay together with all of that part of the variant antigen recognized by the heterologous antisera.

A fundamental question left unanswered was what is the structure of the cross-reacting determinant. Is it a region of constant amino acid sequence or, perhaps, a carbohydrate chain? Peptide mapping of tryptic and chymotryptic digests of isolated variant glycoproteins revealed, mainly, the large extent of diversity. Using the technique of comparing composite maps of two antigens run together with their individual maps, we found a small number of overlapping peptides but not sufficient evidence to identify a peptide as the cross-reacting determinant without sequence data on the overlapping peptides together with further immunologic evidence. To discover, therefore, whether attention should be concentrated on the overlapping peptides or on the carbohydrate part of the molecule, we have been investigating the effects of some gross treatments on the ability of variant antigens to cross-precipitate or to inhibit the heterologous immunoprecipitation reactions.

We treated 221 antigen with trypsin, which produced extensive degradation of the molecule as revealed by SDS-gel electrophoresis but did not completely abolish the molecule's ability to inhibit the heterologous precipitation, ^{125}I-221 × anti-RMI. The degree of inhibition was reduced compared to that in the control (Table 2). Treatment of the carbohydrate part of the antigen molecule, however, had more drastic effects.

Because periodate cleaves carbon-carbon bonds of sugar residues containing two adjacent, unsubstituted, hydroxyl groups, we used it to test whether carbohydrates are involved as antigenic determinants. However, periodate is a strong oxidizing agent that could also change the structure of the protein. For example, it may oxidize the amino acids serine and threonine.

Periodate treatment of 221 antigen, with 250 moles/mole glycoprotein for 24 hours, completely destroyed the ability of the antigen to inhibit the heterologous reaction ^{125}I-055 × anti-221. It also abolished the antigen's ability to bind with Concanavalin A but not its ability to react with the homologous antiserum, anti-native 221 (Table 2). We had a similar result when we treated a second surface antigen, RMI, with the same amount of periodate. The fact that Concanavalin A binding is altered by periodate treatment signifies that some carbon-carbon bonds in the sugar residues of the glycoproteins are susceptible to periodate, because Concanavalin A has binding specificity for mannose and glucose. Briefer treatments with less periodate did not completely destroy the antigen's ability to inhibit. The minimum treatment that still had a dramatic effect on the inhibiting ability of the surface antigen was a periodate-to-glycoprotein ratio of 60:1 and digestion time of 46 hours (Table 2). The resulting periodate-treated 221 antigen (60 moles/mole glycoprotein) was labeled with ^{125}I, by the chloramine-T procedure and immunoprecipitated by homologous and heterologous antisera against untreated surface antigens (Table 3). The modified antigen still precipitated well with the homologous antiserum (anti-221), although less than did the control. The ability to precipitate with a heterologous antiserum (anti-RMI) was almost completely abolished, as expected from the inhibition results.

Table 3. Effect of periodate treatment (60 moles/mole glycoprotein) on immunoprecipitation of ^{125}I-labeled 221 antigen.

Antiserum dilution	% Precipitation	
Anti-221	Control	Periodate-treated
10^{-1}	99	93
10^{-2}	71	47
10^{-3}	39	19
10^{-4}	16	7
10^{-5}	8	4
10^{-6}	5	1
Anti-RMI		
10^{-1}	64	5
10^{-2}	31	2
10^{-3}	12	1
10^{-4}	3	0
10^{-5}	2	0
10^{-6}	1	0

Our conclusion is that cleaving carbon-carbon bonds in the carbohydrate may abolish the glycoprotein's ability to cross-precipitate. The results provide us with a definite stimulus to concentrate further efforts on the carbohydrate and to look for supportive or contrary evidence, for example from the use of specific glycosidase enzymes, or immunoprecipitation from a cell-free protein synthesis system, where the variant antigen should be nonglycosylated.

Mechanisms of antigenic variation in salivarian trypanosomes[1]

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Abstract. Since the early 1900s there has been controversy on whether trypanosomes require stimulation from a host's antibodies to vary their surface antigens. Our studies in vitro and in vivo indicate that elimination of variant types is hastened by the host's antibodies but antigenic variation is not dependent upon them. We cultured and maintained in vitro a total of 14 clones of variable antigen type 052 of *T. brucei* and, using immunofluorescent techniques, detected antigenic variation in 9 of them. Variant type 221 appeared along with other types that were infective to mice but were not recognized by antisera against 052 and 221. Although 052 and 221 have equal growing times in vivo; in vitro 221 outgrows 052 sufficiently to be detected by present techniques. Despite different growing times in vitro, the direction of variation is the same in vivo as in vitro.

As early as 1909, some scientists postulated that during trypanosomiasis the host's antibodies against surface antigens induced the trypanosome to undergo antigenic variation (Ehrlich 1909; Ehrlich, Roehlant, and Gulblausen 1909); others (Levaditi and McIntosh 1909) believed that the antigenic variation was the result of mutational events independent of environment. The controversy is still largely unresolved, but Beale (1954) and Sommerville (1970) have clearly shown that, under in vitro culture conditions, environmental stimuli including antibodies induce change in the surface antigens of several free living protozoa.

Now that it is possible to clone and maintain animal-infective bloodstream trypanosomes in vitro (Hirumi, Doyle, and Hirumi 1977; Hirumi, Hirumi, and Doyle 1978), the processes underlying antigenic variation can be investigated under defined conditions. Previously, the parasite had to be maintained in normal or immunosuppressed laboratory animals. Recently, Cross (1975) isolated and characterized the variant-specific surface glycoproteins of bloodstream trypanosomes, permitting the production of highly specific antisera for use in the antigenic analysis of trypanosome populations. In the past, antisera were derived by infection of a suitable host. These advances together with immunofluorescence techniques that allow analysis of the antigenic type of individual living trypanosomes have enabled us to observe the process of antigenic variation in vitro in the absence of host antibodies (Doyle et al. submitted for publication).

We cultured and maintained in vitro a total of 14 clones of variable antigen type clone 052 of *T. brucei* stock S427 (Cross and Manning 1973; Cross 1975) for up to 60 days and detected antigenic variation in 9 of them. A new variant type (221) appeared in all clones. Also appearing were populations of trypanosomes that were infective for mice but not recognized by antiserum to 052 or 221 type trypanosomes. They probably are a mixture of variant populations to which we do not, as yet, have specific antisera. This phenomenon is similar to antigenic variation in vivo in that variable antigen type 221 consistently appears in the first relapse of mice infected with clones of variable antigen type 052. Again, variable antigen type 221 occurs together with trypanosomes against which we do not have antisera. Thus, antigenic variation can occur in vitro in the same direction as in vivo in the absence of antivariant antibodies.

[1] Supported by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO).

In vivo, mice inoculated with a single trypanosome exhibit new variants in the first relapse, generally 10–12 days after infection, whereas, in vitro, new variants are first detected 18–46 days after initiation of the clones. In the normal host, antibodies eventually remove the original variant population, facilitating detection of new variants; in vitro, where there are no antibodies, the population doubling time (PDT) of trypanosomes is the key to detection. Both 052 and 221 variable antigen types have PDTs of approximately 6 h in vivo, whereas they are 14 h and 8 h respectively in vitro. The marked difference in the PDTs in vitro allows detection of 221 type trypanosomes, which inevitably overgrow the original 052 population to the point of detection. Present techniques are not sensitive enough to detect any new variant type that has a PDT similar to or longer than the original variant.

The difference in PDTs is most intriguing in that both populations originally derived from a single trypanosome and, thus, have the same genotype but different surface antigens.

Differential growth rates of variant populations of *T. brucei* in vivo have been recorded (McNeillage and Herbert 1968; Van Meirvenne et al. 1975), and my colleagues and I have observed the phenomenon in *T. vivax*-infected mice and goats (de Gee, Shah, and Doyle submitted for publication). We examined two variant populations of the same genotype that were poorly infective to mice (at most two parasitemic waves). We found that goats infected with either of the two populations suffered from a relapsing infection and that the trypanosomes appearing in the goats 10–12 days after infection could cause lethal relapsing infections in mice. Immune lysis analysis indicated that the original and the goat-derived populations were antigenically different.

The physiological differences of the variable antigens are poorly understood but may be of great importance in elucidating the genetic mechanisms underlying antigenic variation. It may be that *T. vivax* infections in mice are close to *T. brucei-rhodesiense* infections in which preliminary evidence suggests a correlation between a clone's acquisition of a given variable antigen type and its ability to infect humans (Van Meirvenne, Magnus, and Janssens 1976).

Another area of parasite physiology and host/parasite relationships may prove to be relevant to our understanding of the phenomenon of antigenic variation: the ability of *T. brucei* to undergo a complex series of physiological changes during the course of a parasitemic wave. Whereas the changes apparently adapt the trypanosomes for onward transmission to the tsetse fly, they preclude further multiplication in the mammalian host (Vickerman 1971; Vickerman and Tetley p. 23). While the majority of parasites in a parasitemic wave are undergoing this physiological shift, the trypanosomes carrying new variable antigen types are able to continue multiplying in the mammalian hosts. How this phenomenon relates to the process of antigenic variation is at present under study.

Although the process of antigenic variation is complex, it does not require the action of host antibodies to induce it. Whether or not other physiological stimuli are involved is uncertain, but the occurrence of other complex physiological changes in bloodstream trypanosomes suggests that the switch to display a new variant surface antigen is only part of a far larger process.

Genetic basis of antigenic variation

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Abstract. Antigenic variation in trypanosomiasis is the sequential expression of a series of antigens on the surface of the parasites. How many variants a single cell can express and how variation occurs are at present unknown. Nucleic acid hybridization studies have revealed that the messenger RNA (mRNA) coding for the variant-specific surface antigen is an abundant mRNA species. They also indicate that the abundance of the antigen mRNA is unique to each trypanosome clone but that the sequence is not unique and is found at low levels in unrelated cell clones. Possibly, antigenic variation is a selective increase of transcriptional activity of different antigen genes, and other mechanisms, such as the DNA/gene arrangement, may contribute to the highly mutable character of trypanosomes.

Antigenic variation in trypanosomiasis (reviewed by Barry and Hajduk p. 51) is the sequential expression of a series of antigens on the surface of the parasites. In a normal host, the initial population of parasites derived from a single cell uniformly expresses a single membrane surface antigen. Subsequent parasitemias rise and fall in waves, each new parasite population carrying a new membrane surface antigen immunologically distinct to previous antigens. Only recently has work been devoted to the biochemical characterization of the antigens involved in the antigenic variant (see Cross p. 32; Barbet et al. p. 38). The trypanosomes carry on their external membrane surface a single predominant glycoprotein that carries the immunospecificity for each variant trypanosome population. The surface glycopeptide constitutes 90% of the total membrane protein and as much as 10% of the cell protein by weight. The pure variant-specific surface antigens have similar molecular weights, ranging from 57 000 to 65 000 daltons. The antigens differ greatly in isoelectric points and are rapidly synthesized by intact cells in vitro. Amino acid sequence analysis of four variant antigens shows that the 30 amino acid residues on N-terminus bear no relationship to each other. My colleagues and I have begun to investigate how antigenic variation occurs. We purified the messenger RNAs coding for a variety of variant antigens and studied the mechanisms of expression of the variant antigen structural genes. We then used the pure RNAs in hybridization studies to analyze the genetic basis of antigenic variation.

In our original hybridization study on *T. brucei*, we used complementary DNA probes synthesized from purified mRNAs to analyze the sequence complexities of the populations (Williams et al. 1978). The complexity measurements of the total messenger RNA population of cloned trypanosomes indicated the presence of abundant and scarce RNA sequences. We determined that approximately 20% of the total cytoplasmic messenger RNA population has a sequence complexity of 9.6 kilobases. This would be equivalent to three or four messenger RNAs averaging 2 kilobases long. This quantity of abundant RNA sequences in the cytoplasm of the trypanosome is unexpected and compares with such unusual cell populations as myeloma cells where the predominant protein synthetic activity in the cell cytoplasm is producing one cell protein, namely, immunoglobulin.

To analyze the sequences further, we compared two unrelated cell clones of trypanosomes. We synthesized complementary DNA from messenger RNA of a clone of cells isolated from strain LUMP 227 and hybridized it to RNA purified from a clone of trypanosomes isolated from strain S427. The object of this experiment was to determine whether the two unrelated clones had any sequences in common. Our data indicated that the abundant RNA sequences were clone specific. That is to say,

the abundant sequences were unique to each clone that we tested. We reasoned that the specificity in abundant RNA sequences was due to the variant antigen messenger RNA because the variant antigens are the only readily observable difference between cell clones of *T. brucei*. The presence of variant antigen mRNAs in the population is suggested also by our finding that one of the major proteins synthesized in a cell-free-protein synthesizing system was the variant antigen.

All the complementary DNA hybridized with the RNA from the unrelated clone of the trypanosomes, indicating that although the abundance of the messenger RNA sequences is clone specific, the sequences are present in both clones. Because the RNA was isolated from intact polysomes, it appears that the RNAs for different variant antigens are not only transcribed but also translated in each cell clone. If this is true, the translated variant antigen proteins will not be detectable either by purification techniques to separate contaminants in the variant antigens or by immunofluorescent techniques to measure trypanosome clone integrity. At any rate, these techniques are not as sensitive as the hybridization techniques used in this study. The result suggests that at least some of the genes coding for the low complexity or abundant RNA sequences in independently isolated *T. brucei* clones are not clone specific but that their mode of expression is clone specific.

In the antigenically unrelated *T. brucei* clones, the specificity of expression may be the result of factors that modulate gene activity and define mRNA function. Our result indicates that the genes for several, or possibly many, variant antigens are transcriptionally and translationally active at the same time in the same cell and that antigenic variation is perhaps a selective increase of transcriptional activity of different trypanosome antigen genes. In addition to transcription, a change in the rate of RNA processing or a change in mRNA half-life may contribute to the change in mRNA abundance and to the appearance of new variant antigens.

In view of the large number of variant antigens observed in the field and in the laboratory, we believe there are other mechanisms contributing to the polyphenotypic character of the trypanosome surface antigens. Because the variant antigens are all restricted to a relatively small molecular weight range (57 000–65 000 daltons) and all are located on the exterior of the cell membrane, they may be encoded in the genome DNA as a multigene family. Nucleotide sequence homology between the variant antigens has yet to be demonstrated, but the proteins seem to exhibit the other three properties of a multigene family, i.e., multiplicity, close linkage, and related or overlapping phenotypic function (Hood 1976). If our data are correct, i.e., if unrelated *T. brucei* strains have several or many variant antigens in common, a multigene family seems possible.

As there is no conclusive evidence for any specific mechanism, a look at several other highly mutable or polyphenotypic gene systems may be worthwhile. One of the most obvious systems that produces large numbers of variant proteins is the immunoglobulin gene family. This system comprises a gene family that is limited in numbers but is able to produce extremely large numbers of different proteins. Differentiation of antibody-producing cells results in DNA rearrangement such that the constant and variable genes of immunoglobulin molecules are moved closer together in the genome. The rearrangement of different constant and variable genes can contribute to the production of immunoglobulins with totally different immunospecificities. Such a system is possible also in the trypanosome, although some data refute it. Recent data on immunologic cross-reactivity of different variant antigens (see Barbet et al. p. 38) suggest that the different variant antigen polypeptides do not share any large region of amino acids. Nevertheless, DNA/gene rearrangement may contribute to the production of a large spectrum of antigens.

Another mechanism responsible for genetic instability and an apparent high rate of mutation is the transposition of genetic elements (reviewed by Nevers and Saedler 1977). Insertion sequences (IS) in bacteria and controlling elements in *Zea mays* (Fincham and Sastry 1974) are small pieces of DNA that are capable of inserting themselves into genomic DNA and disrupting the expression of the gene. The IS DNA sequences are capable of inserting and excising themselves and producing varied frequencies of mutant expression and recombination. Depending on how and where the sequence is inserted, the inserted genetic elements may cause different gene products from a single gene, a total shutdown of the gene transcription, the production of modified gene products or entirely new and unrelated gene products if the insertion sequence disrupts a controlling or organizer gene. Similar types of DNA insertions are thought to be responsible for chromosomal rearrangements and mutations of the highly variable white eye locus in *Drosophila melanogaster* (Green 1975). The gene inversions, deletions, and transpositions may create new nucleotide sequences or even new genes at their fusion points.

The importance of large-scale DNA/gene rearrangements in explaining the creation of a diverse array of antibodies and the observation of very highly mutable or unstable genes may have direct relevance to an understanding of the large number of variant antigens produced from a single trypanosome or from a strain of trypanosomes. The only way to elucidate variant antigen diversity is with the recent technique of DNA cloning in bacteria.

Cyclical transmission and antigenic variation

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Abstract. To demonstrate the heterogeneity of metacyclic forms of salivarian trypanosomes, an investigator must test forms that are homogeneous with regard to maturity (surface coat) and, therefore, infectivity. Metatrypanosomes must be kept stable at 0–2 °C throughout the tests to avoid any change in VATs. Bearing these prerequisites in mind, my colleagues and I obtained freshly extruded salivary forms of *T. (T.) brucei* and passed them through a DEAE-cellulose column to separate the mature forms from the procyclic proventricular and epimastigote forms as well as immature metacyclics. We prepared antiserum and tested it on fresh metacyclics before and after DEAE separation. Using the indirect immunofluorescent antibody test and the neutralization of infectivity test, we found that the antiserum effectively neutralized the infectivity of both populations, although the saliva forms before separation exhibited some heterogeneity.

In 1940, Broom and Brown first described antigenic similarities between "cyclical sub-strains" growing in rats that had been infected with different variant antigen types (VATs) of a strain of *T. brucei*. Later, Gray (1965) cyclically transmitted a strain of *T. brucei* and found an early common bloodstream VAT ("basic predominant strain antigen") in different hosts. In extending this work, Gray (1975) used *T. gambiense* isolates and showed that tsetse flies may transmit trypanosomes of more than one antigenic type. Whereas Broom and Brown had used the red cell adhesion test to identify the VATs present in the trypanosome populations, Gray used the agglutination test.

An attempt was also made to study the VATs of metacyclic forms. Cunningham (1966) used the neutralization test to examine cryopreserved metacyclics of *T. rhodesiense* strains and found that metacyclic VATs from several flies infected with the same strain were similar. At least two different VATs were observed; no cross-reactions occurred between metacyclic forms of different strains.

In a recent study, Honigberg et al. (1976), using quantitative fluorescent antibody methods, found no similarity between VATs of metacyclic forms and the corresponding bloodstream forms in mice.

My colleagues and I (Jenni 1977a, b) examined fresh metacyclic forms from flies infected with different VATs of a strain (and cloned derivatives) of *T. brucei* and found them antigenically identical. We carried out serological analysis by applying the neutralization of infectivity test (NIT) in parallel with the indirect immunofluorescent antibody test (IFAT) on living metacyclics in suspension and found no similar VATs among different *T. brucei* isolates. We observed that the metacyclic forms underwent rapid antigenic change immediately after they transformed to the long slender bloodstream form in the mammalian host.

In contrast, Le Ray, Barry, and Vickerman (1978) reported antigenic heterogeneity of salivary gland forms of a strain of *T. brucei*. They conducted immune trypanolysis and indirect immunofluorescent antibody tests. Their findings agreed with those of Vickerman (1978) who studied the reactions of monospecific antisera. He noted heterogeneous reactions when antisera to characterize bloodstream VATs were applied to air-dried and acetone-fixed saliva trypanosomes.

Whether or not the strains are antigenically heterogeneous is difficult to resolve because hyperimmune antisera against metacyclic VATs are almost impossible to obtain. To produce these antisera, surface coat antigens are isolated from metacyclic forms (Cross 1975) that must be harvested from tsetse flies. The problem is in collecting sufficient numbers of metacyclic forms. Monoclonal antibodies against metacyclic VATs are easier to obtain and may prove valuable for analyzing surface coat glycoproteins of metacyclic populations.

To demonstrate antigenic heterogeneity, an investigator must serologically test salivary forms that are homogeneous with regard to maturity (surface coat) and, therefore, infectivity. Furthermore, the freshly extruded metatrypanosomes have to be kept in stable conditions at 0–2 °C throughout the tests to avoid any change in VATs and subsequent infectivity for the mammalian host (Jenni and Brun 1977).

In a recent experiment (Jenni in preparation), we obtained freshly extruded salivary forms of *T. (T.) brucei* STIB 247 (a recent field isolate from a hartebeest, Geigy and Kauffman 1973) from 25 infective flies (*Glossina m. morsitans*) and passed them through a DEAE-cellulose column (Lanham and Godfrey 1970). Only the mature metacyclic forms passed through the column; the procyclic proventricular forms, epimastigote forms, and immature metacyclics were retained. The homogeneous metacyclic population was counted and irradiated with 60 krad at 0–2 °C by a ^{137}Cs source.

A New Zealand white rabbit received 1.5×10^5 metatrypanosomes three times, at weekly intervals, intradermally, with no additional adjuvant. The rabbit was bled 1 week after the third injection and the resulting antiserum was tested on fresh metacyclic forms before or after separation on DEAE cellulose. The IFAT in suspension and the NIT were applied to both populations. Although the antiserum effectively neutralized the infectivity of both populations, some heterogeneity (unstained, faintly stained, and brightly stained forms) was observed in the saliva forms before DEAE separation and was apparent in air-dried and acetone-fixed saliva probes from trypanosome-carrying flies. No fluorescent activity was detected on midgut and epimastigote forms obtained from dissected infected flies. These results strongly suggest that mature metacyclic forms revert to a limited number of VATs, if not to a single VAT.

To date, considerable information on cyclical transmission has become available for the subgenus *Trypanozoon*; the same cannot be said for the subgenera *Nannomonas* and *Duttonella* (Doyle 1977).

In one study, Nantulya, Doyle, and I (1978) carried out the antigenic analysis of metatrypanosomes of *T. congolense* by using the IFAT on tsetse saliva probes and the NIT. We found that different bloodstream VATs of one genotype reverted to a characteristic metacyclic antigen type. VATs from different genetic backgrounds showed heterogeneous metacyclic VATs after simultaneous cyclical transmission through *G. m. morsitans*. Metacyclics of different primary isolates carried different surface variant antigens.

The antigenic composition of *T. vivax* metacyclic forms is at present unknown.

Acknowledgments

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Antigenic heterogeneity of bloodstream and metacyclic forms of *T. brucei*

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Abstract. Heterogeneity of variable antigen type (VAT) in bloodstream populations of *T. brucei* has been widely reported. The mechanism allows the parasite to persist in an immunized host. To determine whether heterogeneity extends back to the metacyclic population, which *Glossina* introduces into mammals, we extended previous studies (Le Ray, Barry, and Vickerman 1978) that demonstrated metacyclic VATs expressed by bloodstream forms. Using a syringe-passaged line of trypanosomes, we isolated clones from a rabbit at the onset of lytic activity against metacyclics. Monospecific antiserum to one of the clones (with VAT AnTat 1.30) showed lytic and immunofluorescent activity against 11–20% of metacyclics. Another VAT, AnTat 1.6 accounted for 8–10% of metacyclics. The significance of the results to vaccination and the genetic control of antigenic variation is discussed.

When the tsetse fly introduces metacyclic forms of trypanosomes into the mammalian host, it initiates the pathogenesis of trypanosomiasis. The trypanosomes not only manage to keep ahead of the host's immune response but also cause, directly or indirectly, the gross pathological effects characteristic of the disease. The most obvious and most studied means by which they evade the host's response is antigenic variation.

To date, most studies of antigenic variation have been done on the subgenus *Trypanozoon*. Because of its high infectivity and virulence in mice and its ease of handling in vitro, it is more suitable for laboratory study than are the economically important *Duttonella* and *Nannomonas*. For similar reasons, most studies have focused on the trypanosome in the mammalian bloodstream.

Syringe-Passaged Populations

In the past decade, trypanosome clones have been extensively isolated and used to analyze antigenic variation during single bloodstream infections (McNeillage, Herbert, and Lumsden 1969; Van Meirvenne, Janssens, and Magnus 1975; Van Meirvenne et al. 1975; Capbern et al. 1977). With syringe-passaged trypanosomes, investigators found that each trypanosome possesses a variable antigen type (VAT)[1] conferred by the glycoprotein antigen in its surface coat. Their results indicate that a parasitemic peak is usually a mixture of VATs in different proportions (McNeillage, Herbert, and Lumsden 1969; Van Meirvenne, Janssens, and Magnus 1975). The host eliminates the major type or types at each remission, leaving others (heterotypes) to multiply and form the next peak. During multiplication, the parasites may compete, some growing faster than others, to establish the major VATs of the next peak

[1] The terminology used here (e.g., VAT) conforms to that agreed by an international discussion group (WHO 1978). A subsequent meeting on VAT nomenclature has now extended the system proposed by Lumsden, Herbert, and McNeillage (1967) to include the VAT repertoire of each trypanosome. Thus the series of VATs originally designated AnTat 1–13 (Antwerp *Trypanozoon* antigen type 1–13) (Van Meirvenne, Janssens, and Magnus 1975) are now designated AnTat 1.1–1.13, meaning that these are VATs 1–13 in the VAT repertoire 1 studied in Antwerp. This repertoire also has a new code: AnTAR 1 (Antwerp *Trypanozoon* Antigen Repertoire 1).

(McNeillage and Herbert 1968; Herbert 1975; Van Meirvenne, Janssens, and Magnus 1975). The existence of minor variants in a peak is clearly important for continuation of the infection and, in keeping with this, antigenic variation would appear to occur spontaneously, without being induced by specific antibody (Van Meirvenne, Janssens, and Magnus 1975; LeRay et al. 1977; Barry in press).

Certain VATs tend to predominate early in infections (Gray 1965; Van Meirvenne, Janssens, and Magnus 1975; Capbern et al. 1977), emerging in a loosely defined sequence regardless of the infecting VAT (Capbern et al. 1977). The number of VATs that a single trypanosome can express is known as its VAT repertoire, and although the extent of this is as yet unknown, Capbern et al. (1977) have shown that one clone of *T. equiperdum* produced at least 101 VATs. Within the subgenus *Trypanozoon*, different repertoires have common or cross-reacting VATs (Van Meirvenne et al. 1975; Van Meirvenne, Magnus, and Vervoort, 1977).

The overall picture is of the trypanosome expressing a large number of VATs in a loosely defined sequence. Presumably, the greater the number of VATs at any one time, the better the chance of the infection's persisting. Thus, a high degree of heterogeneity in the trypanosome population seems to be more important than the individual parasite responding to changing host conditions.

T. vivax can now be cloned, and similar studies on antigenic variation can be applied. We have intravenously injected into fresh mice single organisms of a naturally mouse-infective line isolated by Leeflang, Buys, and Blotkamp (1976). Ten clones, including 6 VATs, have been isolated. Interestingly, none of these shows antigenic cross-reaction with any of 9 *Trypanozoon* VAT repertoires investigated by Van Meirvenne, Magnus, and Vervoort (1977).

Preadaptation of Metacyclics

Some findings on the biology of metacyclics are relevant to this study of their antigenic variation: for instance, initiating infections with single metacyclics is relatively easy (Le Ray, Barry, and Vickerman 1978). In one experiment five mice were injected intravenously and five intraperitoneally with single metacyclics of *T. brucei* obtained by allowing a single tsetse fly to probe into guinea pig serum. In the intravenously injected group, all five developed infections, whereas only three in the other group were infected. The course of parasitemia of the intravenous clones was 1 day in advance of the intraperitoneal clones (Fig. 1), demonstrating clearly the metacyclics' high infectivity to mice. As previously hypothesized by Vickerman (1969b), the metacyclics appear to be preadapted for life in the bloodstream without needing to adapt extravascularly in the chancre before invading the vascular system.

Cyclically Transmitted Populations

To examine antigen variation in populations arising in mice following cyclical transmission, Le Ray et al. (1977) passaged AnTAR 1 trypanosomes (Van Meirvenne, Janssens, and Magnus 1975) through *Glossina morsitans* and used cloning, immunofluorescence, and trypanolysis to identify the ensuing VATs. Cloning of trypanosomes (derived from mice bitten by single infective flies) yielded 13 clones of which 3 were of types previously identified within the series AnTat 1.1–1.13; the remainder (10) included 8 new types.

Two features were immediately evident. Firstly, the cyclically passaged material provided clones that were less virulent and antigenically much less stable than did the syringe-passaged material

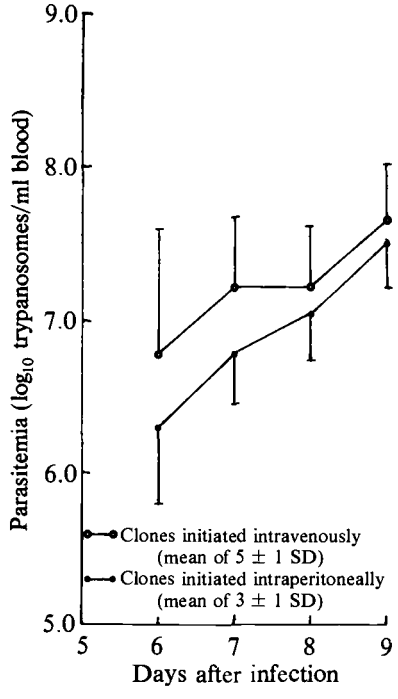

Fig. 1. *Parasitemias (estimated by the method of Herbert and Lumsden 1976) for* T. brucei *metacyclic clones.*

Table 1. Antigenic instability of clones from fly-transmitted populations.[a]

Clone	Passages from cloning (d)	Variants (%)
1	3.3.3.3.3	94
4	7.3.3	14
8	7.3.3	<1
9	7.3.3	6
11	7.3.3	5

[a]Variants were detected in trypanolysis using pooled antisera against AnTAR 1 trypanosomes (based on Le Ray et al. 1977).

studied by Van Meirvenne, Janssens, and Magnus (1975) (Table 1). The differences were detected in populations rather than individual trypanosomes and may reflect differences in degree of pleomorphism (Le Ray et al. 1977; Barry, Le Ray, and Herbert in press). To obtain VAT-specific antisera against the new clones, Le Ray et al. (1977) adapted the trypanosomes to mice by repeated syringe passage, neutralizing heterotypes at every 3-day passage. By this method, specific antiserum was raised successfully for most of the VATs.

Secondly, the populations arising in mice after fly bite proved to be antigenically very heterogeneous, possessing in one case at least 13 VATs after 5 days and 19 of 22 sought after a further 3-day syringe passage in a mouse (Le Ray et al. 1977). Whether the heterogeneity resulted from the antigenic instability of cyclically transmitted trypanosomes or from heterogeneity among metacyclics was not clear, but the general results support the idea that *T. brucei* presents an antigenic mixture to its host: the population is of greater importance than the individual trypanosome.

Metacyclic Populations

To look at the VATs of metacyclics, Le Ray, Barry, and Vickerman (1978) used a variety of sera in trypanolysis and immunofluorescence on freshly probed metacyclics of VAT repertoire AnTAR 1. They allowed flies to feed on rabbits and after 7 days obtained antisera that were capable of lysing all or most metacyclics in one sample (Table 2). Immunofluorescence gave comparable results. This method of obtaining antiserum is similar to that used by Jenni (1977a) and gives similar results. However, it is completely unsuitable for use in identifying individual antigen types: if the immunizing metacyclics are antigenically heterogeneous, the antiserum will be correspondingly heterospecific, with activity against most or all metacyclics.

Use of more characterized antisera produces a completely different picture (Le Ray, Barry, and Vickerman 1978). Pooled monospecific antisera against AnTat 1.1−1.22 lyse less than 10% of metacyclics in a preparation where up to 100% are lysed by other antisera. Quite clearly then, the metacyclics within one probe are antigenically heterogeneous. Le Ray, Barry, and Vickerman found that all the metacyclics in single probes are lysed with long-term sera from rabbits infected by syringe with bloodstream forms. The significance of these findings is that the metacyclic VATs are not specific to the fly stage of the life cycle; they are expressed also by bloodstream forms.

To demonstrate unequivocally that metacyclic VATs are heterogeneous, we needed to identify at least two types within one probe. The cyclically transmitted trypanosomes, being antigenically unstable, are unsuitable for the production of VAT-specific antisera against clones of either

Table 2. Homology of metacyclic VATs with bloodstream form VATs.

		Trypanolysis (no. lysed / total)		
		Antisera against:		
Fly	Control	Metacyclic[a]	Bloodstream VATs[b]	Bloodstream VAT repertoire[c]
A	0/14	14/16	3/10	7/10
B	0/10	65/76	1/12	10/11
C	0/6	7/8	3/6	–
D	2/100	–	14/169	70/70
D	0/25	–	0/25	50/50
Total	2/155	86/100	21/222	137/141
%	1.29	86.00	9.45	97.16

[a]Rabbit serum 7 days after bite by infective flies.

[b]Pooled monospecific antisera against AnTat 1.1−1.22.

[c]Pooled long-term infection sera (32−43 days) from five rabbits infected with bloodstream form AnTAR 1 trypanosomes.

Source: Le Ray, Barry, and Vickerman (1978).

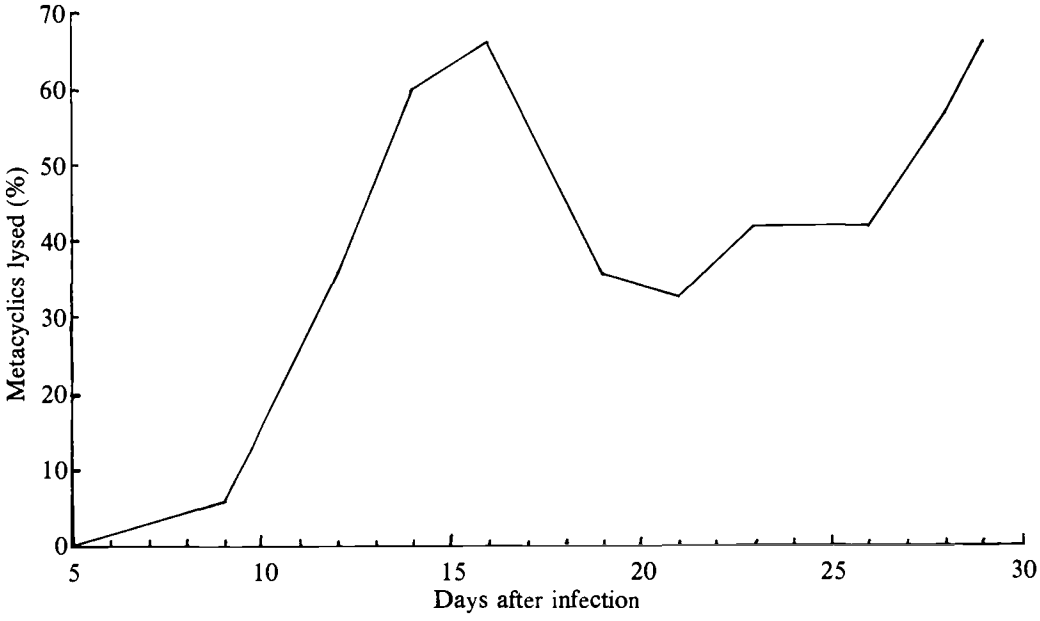

Fig. 2. Development of antimetacyclic activity in a rabbit infected by syringe.

bloodstream forms (Le Ray et al. 1977) or metacyclics (Le Ray, Barry, and Vickerman 1978). Trypanosome lines adapted to mice by at least 20 syringe passages are more stable so we used them to identify and clone single trypanosomes antigenically homologous with metacyclics and subsequently to produce monospecific antisera. The following protocol was devised (Barry et al. in press). We infected a rabbit with a clone from the syringe-passaged line investigated by Van Meirvenne, Janssens, and Magnus (1975). Every 2 or 3 days we took samples of serum and passaged the blood into mice. When parasitemia was patent in the mice, we prepared stabilates in liquid nitrogen. We then tested the whole range of sera in trypanolysis against metacyclics from a single probe and recorded the percentages of metacyclics lysed (Fig. 2). Two or possibly three peaks of activity, none of which reached 100%, were apparent. The finding provides further evidence for antigenic heterogeneity and suggests that at least two or three VATs homologous to metacyclics arose at different times. We initiated cloning, using the stabilate material that was prepared from blood drawn at the onset of the first peak of lytic activity against metacyclics. We tested the VAT of each clone in trypanolysis with antimetacyclic sera taken from rabbits 7 days after fly bite (Table 2).

The 10th clone isolated, whose VAT has now been designated AnTat 1.30 within the AnTAR 1 series, was completely lysed by the antimetacyclic sera. We used it to prepare monospecific antiserum in a rabbit by the method of Van Meirvenne, Janssens, and Magnus (1975) and conducted serological testing on metacyclics. Using immunofluorescence, we found that 11–20% of trypanosomes stained within single probes, and we obtained corresponding figures using trypanolysis. Monospecific mouse antiserum gave the same results — strong evidence for the heterogeneity of metacyclic VATs (Barry et al. in press).

To identify a second metacyclic VAT, we systematically applied to probes 22 monospecific antisera, each against one of AnTat 1.1–1.22, which had previously been shown to account for ca. 10% of metacyclics (Table 1). We expected at least one to exhibit activity. The results indicated that AnTat 1.6 probably accounted for the entire 10%. The conclusion: metacyclics, at least within the AnTAR 1 trypanosomes, are antigenically heterogeneous within a single probe.

The specific antisera against the VATs have also been applied, in a preliminary study, to different probes. Regardless of what VAT was originally ingested by the fly, the age of infection, or the period between probes (at least 2 days), the percentage of the two VATs always fell within certain ranges: 8–10% for AnTat 1.6 and 11–20% for AnTat 1.30 (tables 3 and 4). The behaviour of AnTat 1.30 in a cyclically infected mouse has also been studied: by day 3 post infection 50% of the bloodstream forms were AnTat 1.30. The percen-

Table 3. Presence of AnTat 1.30 and AnTat 1.6 in metacyclic populations.

	Immunofluorescence			
	Anti-AnTat 1.30 (no. labeled/ no. counted)	%	Anti-AnTat 1.6 (no. labeled/ no. counted)	%
Fly				
C[a]	38/200	19	–	–
1	41/220	18.6	17/200	8.5
1	–	–	28/290	9.7
2	81/466	17.4	8/100	8
2	14/100	14	–	–
3	25/140	17.8	20/200	10

[a]Same as fly C in Table 2.

tage decreased on day 4, although the absolute number of AnTat 1.30 rose to its maximum on day 4 and then declined sharply. There was a great increase in numbers of AnTat 1.30 trypanosomes; however, by day 4 they were being outgrown by trypanosomes of a different VAT and were destroyed the following day. The transformation of metacyclics to long slender bloodstream forms would appear in this case not to be linked to antigenic variation. Some of the predominant VATs previously demonstrated by Van Meirvenne, Janssens, and Magnus (1975) were found to grow up as a mixture even from the 4th day of infection.

These data lead to the conclusion, reached before, that *T. brucei* presents an antigenically heterogeneous population as a means of coping with the host's immune system. Thus, individual metacyclics are less important than the whole metacyclic population, within which certain VATs, such as AnTat 1.30, are capable of amplification in the mammalian host and consequent immune destruction. Others may remain at low levels and give rise to the full range of predominant bloodstream VATs. Whether metacyclic VATs can interfere with the expression of each other in the host bloodstream, as postulated for bloodstream populations (McNeillage and Herbert 1968; Herbert 1975; Van Meirvenne, Janssens, and Magnus 1975) remains an open question.

Vaccination

The age-old problem of vaccination merits some discussion in the light of our results. The existence of a single "basic" metacyclic antigen type, and its potential in immunization, must now be viewed as unlikely. A VAT mixture in the tsetse salivary gland appears to be advantageous to the trypanosome, affording greater resistance to the host's immune response. There may be a constant appearance of certain VATs, and at constant percentages, in the metacyclic population; therefore, a "basic VAT repertoire" is possible.

Table 4. Results of immunofluorescence of AnTat 1.30 in sequential probes of one fly.

Age of infection (d)	AnTat 1.30 (%)
26	16
37	20
46	11

If this proves to be the case, vaccination can be reevaluated. Our protocol permits the preparation of large numbers of antigenically stable bloodstream forms of trypanosomes, from which variable antigen can be purified readily (Cross 1975; 1977b) and used effectively for VAT-specific immunization at low concentrations (Baltz et al. 1977). It should also permit the isolation of pure mRNA for potential use in gene cloning in microbial systems and large-scale production of variable antigen (R.O. Williams p. 46).

Of obvious necessity, then, are both the further application of this protocol to characterize fully the VAT complement of probed populations and the determination of the number of serodemes circulating in the field. This approach may be more applicable to *T. vivax* and *T. congolense* than to *T. brucei*, because in these cases substantially fewer trypanosomes, and thus possibly fewer VATs, are probed.

Control of Antigenic Variation

To date, nothing is known about the control of antigenic variation or about the process that initiates the expression of the antigen. Even the demonstration of predominant antigen types does not reveal much, because they are detected as populations and may not reflect what occurs at the level of individual parasites. Nevertheless, our findings on expression of metacyclic VATs may open the way for basic studies on control mechanism(s).

It appears that certain VATs are always expressed in the salivary gland population, and furthermore they are expressed at constant percentages. Although further investigation is required, current evidence suggests that the preinfective epimastigote stage of *T. brucei* is found attached to the secretory epithelium of the salivary gland and gives rise, via immature forms, to the unattached mature infective metacyclic (reviewed by Vickerman 1978). The qualitative and quantitative constancy of these

metacyclic VATs implies that one (or more) of the following conditions exists:

- Up to 20% of epimastigotes are programed to give rise to metacyclics coding for AnTat 1.30 and up to 10% for AnTat 1.6;
- Equal numbers of epimastigotes are programed for eventual expression of different VATs but give rise to metacyclics at different rates;
- Each epimastigote produces 20% metacyclics expressing AnTat 1.30 and 10% expressing AnTat 1.6;
- Metacyclics of different VATs interfere with each other and control their relative numbers in the population, as postulated for bloodstream form VATs.
- The signal for expression is not present in epimastigote forms but is initiated in the immature metacyclic and at different frequency for different VATs.

These possibilities are easy to examine initially just by using monospecific antisera in situ with appropriate markers. Now, there are the means to look at the crucial stage between nonexpression and expression of variable antigen.

Acknowledgments

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Discussion summary

B.A. Newton and K. Vickerman

Understanding the trypanosome organism is the first step in developing measures to control it. Its structure, surface coat, locomotion, secretions, attachment mechanisms, and ability to modify its surface coat all contribute to the pathogenesis of trypanosomiasis. What is known about the organism is little in comparison with what is unknown. For instance, whether or not there are differences in ultrastructures between drug-sensitive and drug-resistant lines of trypanosomes is not clear. To date, no differences have been reported.

Studies of certain forms, such as metacyclics, are hindered by the difficulty in obtaining them. Investigators collecting metacyclics, however, have found that the female tsetse fly produces up to three times as much infected saliva as the male because of her larger salivary glands.

Even the mode of attachment is not completely clear; if the flagellum plays a part in the trypanosome's attachment then it is likely that *T. congolense* attaches in the same way to the proboscis of *Glossina* as it does to small calibre blood vessels.

Although the idea that plasmanemes ("filopodia") from bloodstream trypanosomes attach to red blood cells and blood vessel endothelium is attractive because it would explain the generation of antigen–antibody complexes, there is no good evidence for the production of plasmanemes in vivo much less their adhesion to host cells.

Recently the organism's secretions have received attention. They appear to activate the host's complement system and pharmacologically active substances, such as serotonin and kinins. Although the acid phosphatase secreted by trypanosomes may have a pathogenic effect on the host, it appears simply a convenient lysosome marker enzyme, less important than other enzymes in the pathogenesis of trypanosomiasis.

There may be lipopolysaccharide on the surface membrane of trypanosomes, but the evidence for this is slim. LPS has been reported only in trace amounts in *T. cruzi*; its presence would explain the mitogenic effects of *T. brucei*. Lipopolysaccharide in the serum of trypanosome-infected animals, however, may be a product of gut bacteria absorbed into the blood following increased gut permeability.

There is some evidence that *T. cruzi* produces catecholamines; however, there is little evidence implicating catecholamines in *T. brucei* pathogenesis. In rats infected with the former, there is complete depletion of norepinephrine whereas the latter is associated with only slight depletion of heart norepinephrine.

Surface Coat and Antigenic Variation

The nature of the trypanosome's surface coat and the ability to change the antigenic character of this coat are intriguing problems to scientists studying the

organism. The surface coat appears to be a single glycoprotein; according to Cross and Johnson, the carbohydrate is predominantly located near the C-terminus of the polypeptide chain. Cross (p.32) believes that carbohydrate contributes little to cross-reactions between different antigenic types; however the results of Barbet et al. (p.38) tell a different story. The cross-reactions reported by Barbet et al. are of considerable interest but as yet are not refined enough to be applicable to trypanosomiasis control programs. Experiments in mice and goats so far have not been successful in demonstrating cross-protection between variants.

The carbohydrate may be involved in attaching the glycoprotein to the surface of the cell, possibly through a glycolipid receptor in the membrane. According to another hypothesis of glycoprotein attachment, surface antigen is larger than the molecules that have been isolated, perhaps including a hydrophobic "tail" for insertion into the lipid bilayer. In opposition to this possibility is in vitro work (R.O. Williams) on the biosynthesis of variant-specific glycoprotein that indicates the isolated molecules constitute the entire variable antigen molecule. Furthermore, high resolution gel electrophoresis (T. Pearson) of rapidly lysed cell preparations has not revealed any molecules of greater molecular weight than the purified antigen.

The trypanosome is capable of replacing a surface coat that has been artificially removed, e.g., by capping. Within 3 hours, a new antigen of the same type as was removed appears on the surface. The renewal is not affected by inhibitors of protein synthesis; therefore, the antigen may come from an internal store. How relevant capping studies are to the antibody actions in vivo is questionable.

One mystery of antigenic variation is the number of antigens that can be expressed by a single trypanosome. One study has shown that at least 101 variable antigen types were produced by a clone of *T. equiperdum*. Williams (p.46) suggests that the type is determined by the relative rates of transcription of different mRNAs; and another possibility is that differential translation rates account for the variable antigen type expressed.

The host species, which affects the parasitemic profile of the trypanosome, does not seem to affect its repertoire of antigens, although there is little information on this. Gray's study in 1965 showed a similar antigen sequence in rabbits and goats and is supported by the report of Doyle, Hirumi, and de Gee p.44.

Infections caused by pathogenic African trypanosomes

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Abstract. Potentially pathogenic infections caused by African trypanosomes start when the vector deposits metacyclic forms in the subcutaneous tissues of a mammal. From this site, the trypanosomes are disseminated throughout the host to colonize and multiply in selected tissue microenvironments, which include the circulating blood, capillary beds, and the loose connective tissue stroma of various organs and tissues. It is known that the tissue tropism depends on the species of trypanosomes, but little is known about the relationship between the size of the total population of trypanosomes and levels of parasitemia. Studying *T. vivax*, predominantly a parasite of the circulating blood, we attempted to relate the severity of clinical signs to the levels of parasitemia during infection. We have shown that within uniform groups of Boran and Holstein cattle there is a variation in the severity of clinical disease, varying from a mild asymptomatic syndrome to an acute disease of 4–6 weeks. The most diseased animals had levels of parasitemia that were significantly higher than the asymptomatic subgroup. Even during the first growth phase of parasitemia, significant differences in rate and peak were observed between the two.

This paper deals with the tissue distributions and parasitemias observed in infections caused by the most important species of pathogenic African trypanosomes, namely *T. vivax*, *T. congolense*, and trypanosomes in the *brucei* subgroup, which include *T. brucei*, *T. gambiense*, *T. rhodesiense*, and *T. equiperdum*. The discussion focuses on the colonization by trypanosomes of different tissue microenvironments, the patterns and levels of parasitemia, the relationship of parasitemia to pathogenicity, and the relationship between the populations of trypanosomes in the blood and the total body parasitoses.

Two concepts form the basis for the discussion. The first, emphasized by Dubos (1954, 1955) for bacterial infections and diseases, is that infections by pathogenic trypanosomes do not necessarily result in disease. The second, expressed by Crofton (1971a,b), is that infections are an ecologic relationship between hosts and parasites and must be described in quantitative terms.

In keeping with the aims of the conference, I have compared natural and experimental infections in domestic animals with those in laboratory models.

Site of Infection

The first contact that potentially pathogenic African trypanosomes make with the host is in the subcutaneous tissue at the site of the vector's bite. The initial interaction between the organism and the host at this site has been studied in humans and in some laboratory models infected with organisms in the *brucei* subgroup. The trypanosomes multiply locally in connective tissues and cause a chancre-like skin lesion. The development of similar lesions in domestic animals that have been infected naturally or experimentally with *T. brucei* has not been reported.

Recently, *T. congolense* has been demonstrated, and multiplication postulated, in the connective tissues at the site of injection (Luckins and Gray 1978). To date, it has not been clearly established as to how common and how severe the lesions are in the subcutaneous tissues. Luckins and Gray (1978) refer to three reports that skin lesions have been observed; however, under field conditions, skin lesions are not observed even when animals daily receive very large numbers of bites by infected tsetse flies.

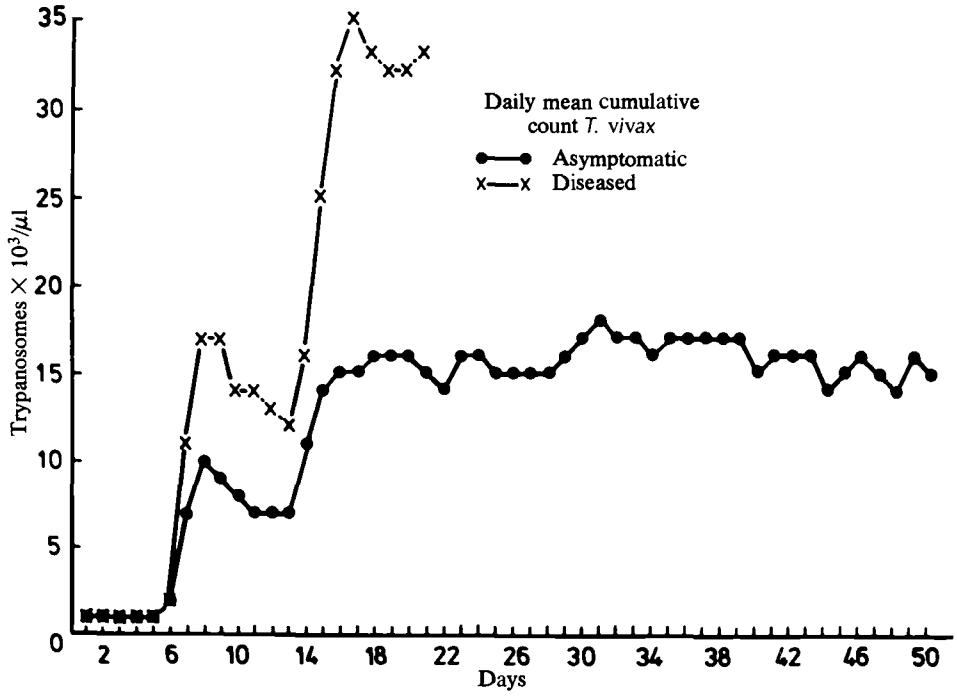

Fig. 1. *Daily mean cumulative numbers of trypanosomes in the asymptomatic and diseased subgroups of Holstein cattle infected with* T. vivax.

There is no available information on the behaviour of *T. vivax* at the site of infection, but because the metacyclic forms of all species are injected by the vector into the subcutaneous tissue, it is likely that the metacyclics and the microenvironment of subcutaneous tissues interact in establishing the infection. From the initial site of infection, the trypanosomes invade the bloodstream, either directly or through lymphatics, causing parasitemia, which disseminates the organisms throughout the circulatory system and, in some species of trypanosome, throughout various solid tissues.

Tissue Distribution

In the study of pathogenesis, one way of grouping African trypanosomes is according to the tissue microenvironments that they preferentially colonize. The observations published in the last 5 years have confirmed earlier findings that the *brucei* subgroup — *T. brucei, T. gambiense, T. rhodesiense*, and *T. equiperdum* — localize in solid tissues, particularly in the loose connective tissue stroma. This is also likely to be the case with *T. evansi*, although there is no published evidence. Tissue localization has been shown to occur in experimental infections of domestic and laboratory animals. In the latter, the tissue invasion is often overshadowed, particularly in the rodent model, by a massive exponential growth of trypanosomes in the circulating blood.

Recent published information has established *T. congolense* as being primarily a parasite of capillary beds. Other than at the initial site, where the vector deposits *T. congolense* in the subcutaneous tissues, the organisms are found only in the blood vessels in domestic and laboratory animals (Losos et al. 1973; Banks 1978; Ssenyonga and Adam 1975).

T. vivax is also primarily a parasite of the circulating blood, although occasionally the trypanosomes have been observed outside the blood vessels (Bungener and Mehlitz 1977). The dominant feature of the distribution of *T. vivax* in the host is that small numbers are rarely observed in connective tissue, whereas large numbers are often seen in the blood vessels. The smaller numbers of *T. vivax* that localize in tissue cause much less damage and inflammatory reaction than do organisms of *T. brucei*, which is an obvious connective tissue parasite with large populations causing extensive lesions in solid tissues. One possible explanation is that *T. vivax* parasites are passively transported into tissues — as opposed to

actively invading tissues — as a result of hemorrhages. The presence of trypanosomes and of hemorrhages in the connective tissues is associated with accumulations of mononuclear inflammatory cells. However, these lesions are neither extensive nor common. The recent isolation of a *T. vivax* strain in West Africa that grows readily in rodents provides a model for further study (Leeflang, Buys, and Blotkamp 1976).

Populations of trypanosomes in the blood in trypanosomiasis syndromes have been studied extensively and play a central role in the study of pathogenesis. There is an underlying assumption in a majority of the studies that the levels and pattern of parasitemia are representative of changes that occur in the total populations of trypanosomes in the body of the host. Unfortunately, there is no published information to substantiate this assumption. Understanding the localization patterns of the trypanosomes necessitates reexamination of the relationships between the parasitemias and the body parasitosis.

Under field conditions, the levels and patterns of parasitemia observed in domestic animals infected with African trypanosomes depend on the species and strain of trypanosomes and on the species of animal infected. All infections caused by trypanosomes are characterized by fluctuations in the level of parasitemia with periodic peaks. The parasitemia levels caused by trypanosomes in the *brucei* group in domestic animals are generally lower than those observed with *T. congolense* and *T. vivax* and are characterized by periodic absence of trypanosomes from the circulating blood. Particularly low

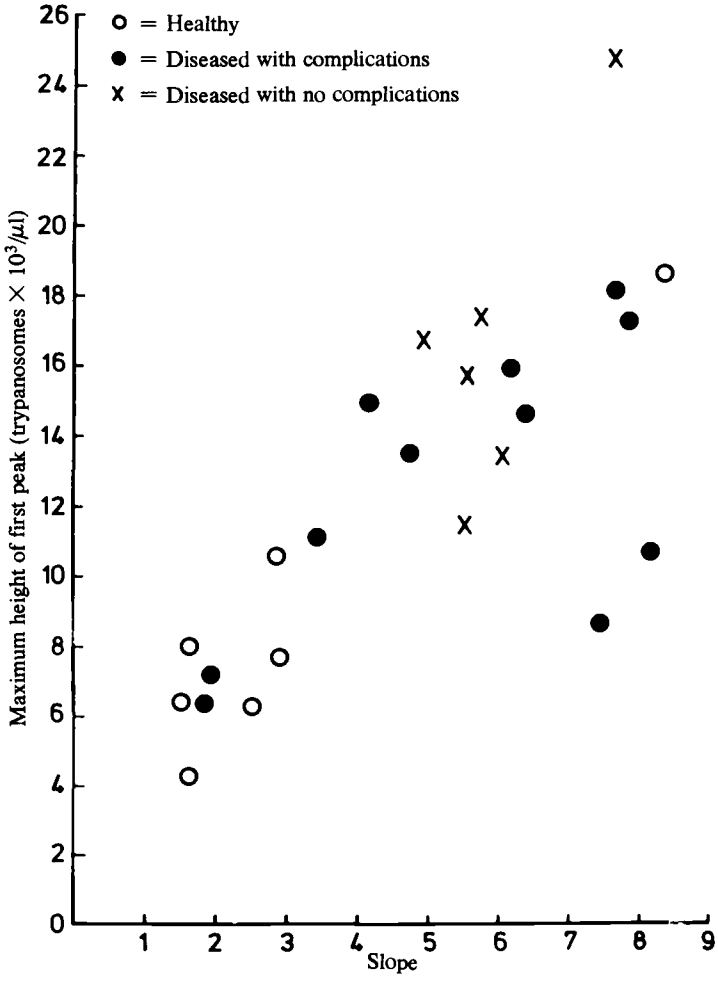

Fig. 2. *Maximum height of first parasitemic peak in 25 Holstein cattle plotted against the slope of the first growth phase of* T. vivax.

parasitemias characterize *T. brucei* and *T. evansi* infections in the bovine, horse, and camel. Highest parasitemias have been observed with *T. vivax*, particularly those strains that are present in West Africa. In infections caused by all species, the levels of parasitemia are highest and the peaks most frequent early in infections. A majority of natural infections in domestic animals are characterized by parasitemias that last for weeks and months. In contrast, infections in some of the animal models are characterized by continuous exponential growth of parasites up to the time of death.

Based on general observations, my colleagues and I postulated a relationship between high levels of parasitemia and virulence in natural and experimental forms of trypanosomiasis. We have undertaken a study on the levels of parasitemia and pathogenicity. Because of the distribution of *T. brucei* and *T. congolense* in tissues, we believed *T. vivax* infections provided the best subject for our studies to establish the relationship between the size of trypanosomal populations and the degree of the host's response. Using large groups of uniform cattle, Boran and Holstein, we found great variation in the clinical responses to a single experimental infection. Some animals die as early as 3–4 weeks after infection, whereas others, identically treated, survive for several months without obvious clinical signs of disease.

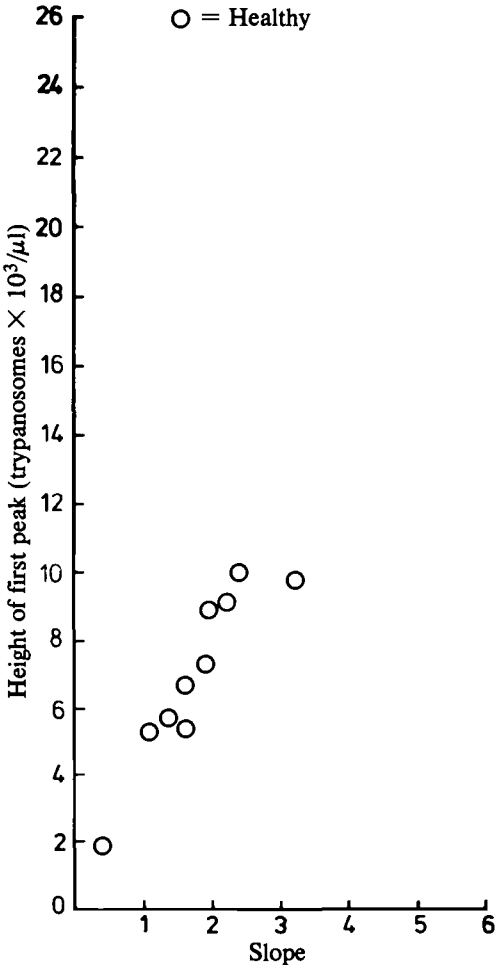

Fig. 3. *Plotted against the slope of the first growth phase of* T. vivax, *the first parasitemic peak in eight asymptomatic Boran cattle.*

Variability of the response of cattle to trypanosomal challenge is a very important feature of experimental and natural trypanosomiasis. This variability must be taken into consideration in experiments, necessitating tests on large numbers of animals. However, the variability of responses has the advantage of enabling the study of a whole range of responses, particularly the comparison of the severe and mild syndromes, in uniform groups of animals. There were obvious differences between the levels of parasitemia in our two groups, designated as asymptomatic and diseased. The daily mean cumulative count was determined by adding daily counts and dividing by the study days. In the diseased group, the daily mean cumulative number of trypanosomes was consistently higher, reaching two to three times higher than those observed in the asymptomatic group (Fig. 1). The doubling time during the first growth phase was approximately 7 hours, a growth rate comparable to that observed with *T. brucei* and *T. cruzi* grown in tissue cultures.

How early in the infection do the differences in parasitemia occur? Plotting the heights of the first peak against the slope of the growth curve during the first cycle for each, we found the two groups are easily discriminated graphically and early (Fig. 2). It appears that this description can be used generally, and in comparable data from other experiments (Fig. 3), in which the response to *T. vivax* infection was classified as asymptomatic, the same area as in Fig. 2 is occupied by the points plotting height of peak against slope.

The different distributions of various species of trypanosomes in the tissues of the host and the differences in the levels and patterns of parasitemia are central to studies of pathogenesis. Because of the differences that are observed in the tissue tropism of various species of trypanosomes, laboratory models have to be selected to resemble more closely the natural host–parasite interaction in disease. In addition, the relationship between parasitemia and the total population of trypanosomes in the body needs to be better defined.

Rodent trypanosomiases

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Abstract. Although the agents of the rodent trypanosomiases are generally considered benign, they can produce severe, and at times fatal, infections in young rodents, in pregnant hosts, and, with concomitant infections, in mature animals. The severity of such infections results from an immature or inadequate immune response. However, even in fully immunocompetent hosts, infections can be associated with splenomegaly, hepatomegaly, anemia, hypocomplementemia, glomerulonephritis, and immunodepression. Although these changes are similar to those produced by the pathogenic species of trypanosomes, they are much less severe and there is a relatively rapid return to normal. This results from the host's immune response, and especially the production of a reproduction-inhibiting antibody, ablastin, which controls the parasitemia until trypanocidal responses eliminate the parasites. In the absence of, or with an inadequate, ablastic response, the rodent trypanosomes may produce fulminating infections strikingly similar to those of the pathogenic species. Like the pathogenic African species, they are capable of antigenic variation, but they are ultimately blocked by the host's development of reproduction-inhibiting immunity, a distinguishing feature of the rodent diseases. An understanding of this unique response, which renders potentially lethal parasites, benign, may be valuable for the control of the pathogenic species.

The rodent trypanosomiases are caused by a large group of parasites that are distributed worldwide and include more than 40 named species of *Trypanosoma* of the subgenus *Herpetosoma* (Hoare 1972). These trypanosomes are generally considered benign or nonpathogenic (D'Alesandro 1970; Hoare 1972; Molyneux 1976; Mansfield 1977). Moreover, with few exceptions, they show a high degree of host specificity and are uninfective for rodents other than the ones in which they occur naturally. These features are in contrast to the African trypanosomes and *T. cruzi*, the agent of American trypanosomiasis, which are considered pathogenic and show broader host ranges (Hoare 1972; Mansfield 1977). Although the appellations "pathogenic" and "nonpathogenic" are convenient designations for these groups of trypanosomes, they are not strictly accurate, for the rodent trypanosomes do harm their hosts and under certain conditions can cause death. Conversely, although the African trypanosomes and *T. cruzi* are usually pathogenic and frequently lethal in man and certain domestic animals, they appear to be well tolerated in many species of wild animals that may also serve as reservoir hosts. Therefore, with both groups of trypanosomes, apparent nonpathogenic infections occur; in the case of the rodent trypanosomiases, this favourable balance has an immunologic basis, but the factors involved in comparable infections with the pathogenic trypanosomes have not yet been identified (D'Alesandro 1970).

In spite of the fairly large number of species of rodent trypanosomes, only a few of them have been studied in detail, and the studies made, though numerous, have usually been immunologic. Studies of the abnormalities produced by these trypanosomes are relatively few and have been confined to two species: *T. lewisi* of the rat (*Rattus* spp.) and *T. musculi* of the house mouse (*Mus musculus*). This limitation has probably resulted from practical considerations, i.e., ease of maintaining laboratory strains of rats and mice. Nevertheless, the information available on these two species will probably be applicable to other members of the group. Moreover, the studies have the advantage that they are based on natural host–parasite relationships.

The pathologic changes in rodent trypanosomiasis have not been studied as

thoroughly as those caused by the pathogenic trypanosomes. It is clear nonetheless, that although they are not usually as severe and extensive as the latter, there are some common features.

Age of Host

Most species of rodent trypanosomes are well tolerated by their hosts, but naturally pathogenic strains of *T. lewisi* that produce lethal infections in rats of all ages have been described by Brown (1914) and have been experimentally developed by rapid passage through rats (Roudsky 1910a, b; 1911a, b). The latter are also infective for mice and other rodents. All strains, however, are more pathogenic for young rats as first reported by Jürgens (1902) and confirmed by others. Severe, and at times fatal, infections with marked anemia were noted in young hosts by Marmorston-Gottesman, Perla, and Vorzimer (1930). In more detailed studies, death rates of 80–90% in rats less than 30 days old were recorded by Herrick and Cross (1936); rats more than 40 days old always survived with few apparent ill effects. Similar results were obtained by Duca (1939), who found that infections killed more than 70% of rats 25 days old or younger in contrast to 6% of animals more than 25 days old. In rats of all ages, however, secondary anemias were observed. Similarly, young mice have been reported to be more susceptible than older animals to infection with *T. musculi* (Culbertson 1941).

The basis of the age resistance of rats to infection with *T. lewisi* appears to be immunologic. Following immunization with formalin-killed blood trypomastigotes, rats less than 25 days old produced low titred agglutinins and showed low resistance to challenge infections, at times succumbing as readily as unvaccinated controls (Culbertson and Kessler 1939). In contrast, older animals readily produced agglutinins and had solid immunities. Further studies (Culbertson and Wotton 1939) showed that young rats were unable to control the reproduction of the trypanosomes, indicating inadequate production of the reproduction-inhibiting antibody, ablastin (Taliaferro 1932). These observations indicate that the generally severe infections of young rodents result from their immunologic immaturity.

Intercurrent Infections

The potential pathogenicity of rodent trypanosomes can be expressed in older, immunocompetent hosts that experience intercurrent infections with other agents. The additional stress imposed on the host by the dual infection can be sufficient to upset the host–parasite balance so that severe or fatal infections occur. *Haemobartonella muris*, which is frequently latent in otherwise normal rats and mice (Marmorston-Gottesman and Perla 1930; Baker, Cassell, and Lindsey 1971; Bartlett and Pease 1975; Cox and Calaf-Iturri 1976; Sogandares-Bernal and Chandler 1978), is most commonly found associated with *T. lewisi*. Such dual infections are especially severe in young animals (Marmorston-Gottesman, Perla, and Vorzimer 1930), but even in mature rats, death rates of up to 50% can occur within the 1st week of the trypanosome infection (D'Alesandro unpublished results). Similarly, *Plasmodium berghei*, which seldom kills older rats, has caused death rates of 50% (Hughes and Tatum 1956) to 68% (Jackson 1959) when injected simultaneously with *T. lewisi*. In contrast, none of the mice simultaneously inoculated with avirulent *P. yoelii* and *T. musculi* died. When trypanosomes were inoculated into mice that had been injected with *P. yoelii* 7 days earlier, however, mortality was more than 60% (Cox 1975). The deaths were attributed primarily to the immunodepressive effects of the malaria on the ability of the host to respond to the trypanosomes. In similar dual infections of mice with virulent *P. berghei* and *T. musculi* (Bungener 1975), all the animals died regardless of the timing of the inoculations, but it was noted that with simultaneously initiated dual infections, the trypanosome parasitemia was stabilized before death.

It is clear from the studies of age resistance and intercurrent infections that an effective immune response is necessary to suppress the potential virulence of the rodent trypanosomes. Nevertheless, even in fully immunocompetent hosts that are able to terminate their infections with little apparent difficulty, some pathogenic changes occur — striking evidence that even under the most favourable circumstances, the rodent trypanosomes are not truly benign.

Splenomegaly and Hepatomegaly

Probably the most obvious abnormality resulting from rodent trypanosomiasis is splenomegaly. In one of the earliest studies, rats infected with *T. lewisi* and *Haemobartonella* had spleens that were six to seven times as large as those in uninfected animals (Marmorston-Gottesman, Perla, and Vorzimer 1930), although it is difficult to determine how much of this increase was due to the trypanosomiasis. In similar studies with

Haemobartonella-free rats Taliaferro, Cannon, and Goodloe (1931) found that *T. lewisi* infections caused only a doubling of spleen size, whereas concomitant infections with *Haemobartonella* resulted in spleens three times the normal; comparable degrees of splenomegaly were reported by Becker, Manresa, and Johnson (1943) and Thoongsuwan and Cox (1978). In one of the most recent studies, presumably in *Haemobartonella*-free rats, about a ninefold increase in spleen size was observed (Ferrante, Jenkin, and Reade 1978). The variations aside, *T. lewisi* clearly induces splenomegaly. Similarly, mice infected with *T. musculi* can have spleens 10 times larger than normal (Albright, Albright, and Dusanic 1977).

Studies of the kinetics of splenomegaly indicate there is a general, positive correlation with the parasitemia. Most of the increase in spleen size occurs within the first 7–10 days of infection with *T. lewisi* and *T. musculi*, and this corresponds to the time of peak parasitemia (Marmorston-Gottesman, Perla, and Vorzimer 1930; Taliaferro, Cannon, and Goodloe 1931; Albright, Albright, and Dusanic 1977), although Ferrante, Jenkin, and Reade (1978) observed maximum spleen enlargement on the 20th day of infection. Parasitemia is terminated shortly after maximum splenomegaly is attained, and the spleen gradually returns to near normal size. Histologically, there is a marked hyperplasia of the white pulp (lymphoid elements) of the spleen so that as the lymphoid follicles increase in size and number, the division between white and red pulp becomes less distinct (Marmorston-Gottesman, Perla, and Vorzimer 1930; Taliaferro, Cannon, and Goodloe 1931; Albright, Albright, and Dusanic 1977; Ferrante, Jenkin, and Reade 1978). There is also hyperplasia of the red pulp (Taliaferro, Cannon, and Goodloe 1931) apparently resulting from erythropoiesis and an increase in lymphoid elements (Ferrante, Jenkin, and Reade 1978). As the spleen returns to normal size, it also regains its normal structure.

Infections with *T. lewisi* and *T. musculi* also cause hepatomegaly, but the liver size does not exceed twice normal (Albright, Albright, and Dusanic 1977; Ferrante, Jenkin, and Reade 1978). In rats, maximum hepatomegaly occurs several days later than the peak of splenomegaly, and the liver returns to normal size more slowly than does the spleen (Ferrante, Jenkin, and Reade 1978); in mice, the liver returns to normal size before the spleen (Albright, Albright, and Dusanic 1977). Histological studies in the rat have attributed liver enlargement to degenerative changes characterized by cloudy swelling of parenchymal cells (Ferrante, Jenkin, and Reade 1978); in addition, there is a marked round-cell infiltration. Ultrastructural studies of liver from *T. lewisi*-infected rats have revealed parenchymal cells with dilated cisternae of the endoplasmic reticulum and swollen and more numerous mitochondria (Simaren 1973; Lee and Barnabas 1974). Hepatocytes with focal areas of lipid and fatty infiltrations were also observed, but normal structural appearance was regained about 5 weeks after infection. Coincident with the general decline and termination of the parasitemia, between the 14th and 21st day of infection, residual lysosomes were found in Kupffer cells, which also contained phagocytized trypanosomes (Lee and Barnabas 1974).

The marked lymphoid hyperplasia and consequent splenomegaly probably result from the intense antigenic stimulation that occurs during infections with rodent trypanosomes. Mitogens of parasite origin may also be involved, as suggested by a recent study (Hazlett and Tizard 1978). Another factor very likely involved is the anemia associated with infection, because erythrophagocytosis has been reported in the spleens of infected rats (Marmorston-Gottesman, Perla, and Vorzimer 1930; Greenblatt 1973; Thoongsuwan and Cox 1978) as well as erythropoiesis (Greenblatt 1973; Ferrante, Jenkin, and Reade 1978).

Anemia

The anemia associated with *T. lewisi* infections of rats has been known for a long time (Marmorston-Gottesman, Perla, and Vorzimer 1930; Duca 1939; Saul and Becker 1949; Barnes 1951; Sherman and Ruble 1967; Tawil and Dusanic 1971; Shaw and Dusanic 1973; Greenblatt 1973; Thoongsuwan and Cox 1978). In some of the earlier studies, concomitant *Haemobartonella* infections complicated the results (Marmorston-Gottesman, Perla, and Vorzimer 1930), but in later work with *Haemobartonella*-free rats, it was clearly demonstrated that *T. lewisi* alone can induce anemia. More recently, anemia has been reported in mice infected with *T. musculi* (Jarvinen and Dalmasso 1977a). In all these studies, the anemia can generally be correlated with changes in the number of parasites and is usually most severe near the time of peak parasitemia. In rats, erythrocyte numbers can decrease from about 7 to 4 $\times$ 10^6/mm^3 (Duca 1939; Thoongsuwan and Cox 1978; D'Alesandro unpublished results) and hematocrit levels from about 45 to 35% (Sherman and Ruble 1967; Tawil and Dusanic 1971; Shaw and Dusanic 1973); similar changes in hematocrit values occur in mice (Jarvinen and Dalmasso 1977a).

The mechanism of anemia in rodent trypanosomiasis is not fully understood. There does

not appear to be a suppression of hematopoiesis because normoblasts and compensatory reticulocytosis occur during the infection (Duca 1939; Greenblatt 1973; Jarvinen and Dalmasso 1977a). Hemolysins of parasite origin have been suggested as the cause of these secondary anemias (Duca 1939), but there is no evidence of intravascular lysis: no hemoglobinuria or hemoglobinemia has been found. Erythrophagocytosis does occur, however (Marmorston-Gottesman, Perla, and Vorzimer 1930: Greenblatt 1973; Thoongsuwan and Cox 1978), and could account for the loss of erythrocytes. Recently Jarvinen and Dalmasso (1977a) have found evidence for an immunologic mechanism of the anemia in *T. musculi*-infected mice. Direct antiglobin tests of erythrocytes were positive: 50% of the mice had only IgG1 on their erythrocytes; the remaining animals had different combinations of IgG2, IgM, IgA, and C3 in addition to IgG1. Because similar degrees of anemia occurred in C5-deficient and normocomplementemic strains of mice and because the presence of C3 in addition to the immunoglobulins on the erythrocytes did not appear to aggravate the anemia, the investigators concluded that intravascular hemolysis does not occur and that C3 is not essential for erythrophagocytosis. Apparently they did not attempt to determine whether parasite antigens were also present on the surface of the erythrocytes, but it is likely that the bound immunoglobulins were part of an antigen–antibody complex that would make the cells susceptible to phagocytosis via one of the mechanisms postulated by Jennings (1976).

The anemia associated with *T. lewisi* infections in rats has not yet been similarly studied, but it may also involve immunologic factors. Exoantigens are produced by *T. lewisi* and remain in the blood throughout the infection (D'Alesandro 1972, 1975; Bawden and Stauber 1974). Also it was noted many years ago by Marmorston-Gottesman, Perla, and Vorzimer (1930) that the serum of *T. lewisi*-infected rats is anticomplementary (i.e., fixes complement), and this finding was interpreted as evidence of circulating antigen–antibody complexes. Possibly, the complexes adsorb temporarily to the surfaces of the erythrocytes and activate complement, thereby promoting opsonization by a mechanism similar to that proposed by Assoku (1975) to explain the anemia in rats infected with *T. evansi*; alternatively, antibody may combine with adsorbed parasite antigens on the erythrocyte surface with subsequent release of the complex. Consistent with these possibilities is the marked reduction in total complement that occurs during *T. lewisi* infections in rats (Jarvinen and Dalmasso 1976; Nielsen, Sheppard, Holmes et al. 1978). In a recent study (Thoongsuwan and Cox 1978), cold-active hemagglutinins for trypsinized rat erythrocytes were detected in plasma from *T. lewisi*-infected rats. However, the role of these antibodies in the anemia is unclear because the induction of similar autoantibodies in uninfected rats did not result in anemia. Nevertheless, other observations argue against an immunologic mechanism. It has been found by Balber (1974), for example, that immunosuppression of mice by treatment with corticosteroids attenuates the anemia associated with *T. brucei* infections. However, treatment with cortisone (Sherman and Ruble 1967) or antilymphocyte serum (Tawil and Dusanic 1971) of *T. lewisi*-infected rats exacerbates the anemias. Possibly, such treatments also affect erythropoiesis, as antilymphocyte treatment alone causes a mild, transient anemia, and cortisone treatment alone, although it causes no anemia, reduces the number of reticulocytes. Furthermore, Greenblatt (1973, 1975) has found evidence of a developmental link between plasma cells and blood cells in his studies of infected rats' spleens and has suggested that because of the intense antigenic stimulus provided by *T. lewisi*, anemia occurs because of competition between lymphopoiesis and hematopoiesis for a common stem cell.

Complement Depletion

Recent studies by Jarvinen and Dalmasso (1976) have shown that a massive activation of complement occurs via the classical pathway in *T. lewisi*-infected rats. Total complement and C4 levels are reduced to less than 10% of preinfection values, irrespective of parasite numbers; C3 levels are inversely proportional to parasitemia, dropping to 35% of normal values with heavy infections; and C6 levels are unaffected. In genetically C4-deficient rats, parasitemias and C3 levels were found to be similar to those of normocomplementemic controls, and the use of cobra venom factor at various times during infection to deplete C3 and late-acting components in C4-deficient and normal rats had no effect on the course of infection. These results indicate that complement is not essential to, or at least does not play a major role in, the control and elimination of the trypanosomes. In contrast, Nielsen, Sheppard, Holmes et al. (1978) found that decomplemented rats developed significantly higher parasitemias than did controls. Their findings may be related to their methods: the cobra venom factor was administered 24 hours before infection with the trypanosomes and in larger doses than in the studies by Jarvinen and Dalmasso (1976), who

suggest that immune complexes are responsible for the complement depletion. It is likely that complement plays a role in the anemia because the level of C3 and the severity of the anemia are both proportional to the parasitemia. Is C3 bound to the surface of erythrocytes of *T. lewisi*-infected rats, and does C3 depletion attenuate the anemia? Unfortunately, these questions have not yet been answered.

In contrast to *T. lewisi* infections, *T. musculi*-infected mice show unchanged or slightly increased levels of C1 and C3 (Jarvinen and Dalmasso 1977b); only in genetically C5-deficient mice are occasional, moderate reductions of these components observed. In normocomplementemic and C5-deficient mice, however, the course of infection is similar (Dusanic 1975b; Jarvinen and Dalmasso 1977b). In addition, treating mice with cobra venom factor late in the infection reduces the rate of parasite elimination and prolongs the infection (Jarvinen and Dalmasso 1977b). Therefore, two closely related species, *T. lewisi* and *T. musculi*, evoke surprisingly different responses in their hosts. The results suggest that elimination of the parasites depends upon complement-mediated opsonization, although, if this is true, a high synthetic rate would be necessary to maintain the generally unchanged levels of complement components observed throughout infection. These essentially unchanged levels, however, are consistent with the conclusion that C3 is not involved in the anemia caused by *T. musculi* (Jarvinen and Dalmasso 1977a).

It has recently been reported that after several hours of incubation at 20 °C, suspensions of *T. lewisi* blood trypomastigotes release anticomplementary factors that can activate bovine, human, and guinea pig complement in vitro (Nielsen and Sheppard 1977); subsequent work has shown that one of the major, active components that can be prepared from cellular homogenates of the parasite is a carbohydrate-rich compound (Nielsen et al. 1977). How these findings are related to the complement depletion that occurs in the natural host is not clear. Rat serum was apparently not tested, and anticomplementary activity was not detected until suspensions of cells had been incubated in buffer for 3.5 h, at which time very few parasites were still viable (Nielsen et al. 1978b). Jarvinen and Dalmasso (1976) found that *T. lewisi* cells can immediately activate rat complement in vitro but only when incubated in serum from immune, not normal, rats. Therefore, although other species of trypanosomes may activate complement directly (See Diffley 1978a, b; Diffley and Honigberg 1978), the available evidence indicates that, during *T. lewisi* infections, complement depletion occurs through the participation of antibody.

The host is especially vulnerable to the rodent trypanosomes during pregnancy when embryonal and maternal death can be common consequences of infection. In the studies of Shaw and Dusanic (1973), it was found that if infections with *T. lewisi* in rats were initiated early in the 1st week of pregnancy, fetal resorption readily occurred and parasitemias were similar to those of nonpregnant controls; in rats infected late in the 1st week of pregnancy, however, resorption was more difficult, and half the females died shortly before parturition, even though parasitemias were not elevated. The midterm of pregnancy was found to be the most vulnerable period, for most of the rats infected at this time had extremely high parasitemias and died at parturition (80%) without giving birth. Conversely, during the last week of pregnancy, resistance appeared to be enhanced, for rats infected at this time had significantly lower parasitemias than did controls and produced normal litters. The anemia associated with the infection was exacerbated in all the infected, pregnant rats except in those infected during the last week of pregnancy. It was also found that the placentas of rats infected at midpregnancy harboured an unusually large number of multinucleated parasites that contained 8–16 nuclei and kinetoplasts, but parasites were not found in fetuses. The mechanism of fetal death is unknown but does not appear to be hormone depletion (Shaw and Quadogno 1975).

Similar observations have been made by Krampitz (1975) in his studies of pregnant mice infected with *T. musculi*. Here, too, the host was most vulnerable during the midterm of pregnancy (between the 4th to 14th days) when fetal resorption, abortion, and maternal death frequently occurred and parasitemias were 10-fold greater than normal; when infections were initiated during the last week of pregnancy, however, parturition and parasitemias followed a regular course. The focus of the enhanced parasitemias appears to be the placenta where immense masses of reproducing trypanosomes are found in unusually large rosette formations. Krampitz (1975) has observed that parasitemias show an immediate decline following normal delivery, abortion, or the surgical removal of the pregnant uterus; extirpation of the embryos alone with preservation of the placenta in situ has no marked effect. Krampitz (1975) suggested that an immune response develops during the susceptible period but is somehow held in abeyance by hormonal or other factors until the uterus is emptied. It is also possible that there is simply an excess of antigen relative to circulating antibody at this time, and the balance is shifted to antibody

excess, with consequent removal of the parasites, when the focus of intense antigen production is removed. That such severe infections also occur naturally is indicated by the intense parasitemias found in wild, pregnant house mice by Krampitz (1975).

Immunodepression

Albright, Albright, and Dusanic (1977, 1978) have convincingly demonstrated that immunodepression can occur in rodent trypanosomiasis. They found that in mice infected with *T. musculi* there is a marked correlation between the kinetics of parasitemia and splenomegaly and the depression of humoral immune responses (see also Hazlett and Tizard 1978). At the time of maximum splenomegaly (day 14), the in vivo response to sheep erythrocytes was 10% of normal, and the in vitro response of infected spleen cell cultures was completely suppressed; following the termination of the parasitemia and the return of the spleen toward normal size, immune responsiveness was regained. Analysis of cell types in infected spleens showed that although the normal ratio of T cells to B cells was doubled at maximum splenomegaly, their absolute numbers were actually increased 10-fold and 5-fold, respectively, because of the hyperplasia. Paralleling the depressed humoral antibody response was a virtually complete suppression of the response of infected-spleen cell cultures to T-cell and B-cell mitogens. Preliminary experiments indicate that humoral substances directly mediate the immunodepression: it was observed that serum from infected mice, saline extracts of blood trypomastigotes, and living blood trypomastigotes ($>10^3$ cell/ml) strongly inhibited the humoral antibody response of normal spleen cell cultures; marked inhibition also occurred when such cultures were separated from the living parasites by membranes with a pore size of 0.22 μm. Whether *T. lewisi* or other rodent trypanosomes have similar immunodepressive effects is at present unknown.

Glomerulonephritis

Viable, infective stages of *T. musculi* can be found in the kidneys of immune mice almost 1 year after termination of parasitemia (Viens et al. 1972; Targett and Viens 1975). How this prolonged presence relates to pathologic changes is not clear because the kidneys, although they become enlarged (up to twice normal), return to normal size about 1 month after infection (Albright, Albright, and Dusanic 1977). Ultrastructural studies have shown, however, that at the peak of parasitemia, when glomerular diameters increased two or three times and trypanosomes were present in glomerular capillaries, there was an infiltration of the glomeruli by eosinophils, neutrophils, and other leukocytes (Molyneux, Kaddu, and Suzuki 1973; Molyneux 1976). The changes associated with glomerulonephritis were not found until 21 days after infection when electron-dense material and a thickening of the basement membrane appeared in glomerular capillaries.

T. lewisi, too, has been found in the kidneys of infected rats (Ormerod 1963, 1975) but, in contrast to *T. musculi*, only during the period of patent parasitemia and not after recovery (Wilson et al. 1973; Targett and Viens 1975). Ultrastructural studies by Simaren (1974) of rat kidneys 14 days after infection have also demonstrated cytopathologic changes, most notably, irregular thickening and splitting of capillary and tubular basement membranes where parasites were localized. In the most recent study, in addition to thickening of Bowman's membrane and tubular basement membrane, hypercellularity of the glomerular tuft, swelling of vascular endothelium and tubular epithelium, and abnormal numbers of hyaline casts were observed (Thoongsuwan and Cox 1978). The etiology of glomerulonephritis in rodent trypanosomiasis is unknown, but very likely immune complexes are involved.

Discussion and Conclusions

Available evidence strongly indicates that the relatively benign nature of the rodent trypanosomiases has an immunologic basis. As pointed out by Taliaferro (1929), the unique aspect of these rodent diseases is the ability of the host to produce a reproduction-inhibiting antibody, ablastin, that controls the parasitemia until trypanocidal responses destroy the parasites. The resulting discontinuous reproductive activity of the rodent trypanosomes contrasts sharply, for example, with the African trypanosomes, which show continuous reproductive activity throughout the infection. The periodic crises observed in the latter, however, are only temporarily effective because of the marked antigenic lability of the pathogenic species. The rodent trypanosomes also undergo antigenic variation, although to a more limited degree (D'Alesdandro 1970, 1976), but the key element in their control is the host's ablastic response. Thus, if the host is adequately immunosuppressed by a variety

of standard methods, an ablastic response does not occur, and the rodent trypanosomes produce fulminating, fatal infections that are strikingly similar to those of the pathogenic species (see D'Alesandro 1970; Dusanic 1975b). Therefore, although both groups of trypanosomes elicit trypanocidal responses, it is apparently the failure of the host to produce an ablastic response against the pathogenic species that allows full expression of their pathogenic potential. Even despite a well developed ablastic response, the rodent trypanosomes cause some pathologic changes, which ironically, are apparently the by-products of the vigorous, effective immune response.

Although the abnormalities associated with rodent trypanosomiases are not generally as severe as those associated with the pathogenic trypanosomes, there are common features. Splenomegaly and hepatomegaly, for example, are clinical features of African trypanosomiasis in humans, domestic animals, and experimental hosts (Apted 1970; Ormerod 1970; Losos and Ikede 1972; Murray, P.K. et al. 1974a; Mansfield and Bagasra 1978); these conditions generally develop quickly and persist throughout the infection. In rodent trypanosomiasis there is a relatively rapid return to normal that parallels the decline and termination of infection. Moreover, the degree of organ enlargement can be much greater with the pathogenic species, at least under experimental conditions. Thus, *T. musculi*-infected mice show a 10-fold increase in spleen size (Albright, Albright, and Dusanic 1977), whereas mice infected with *T. rhodesiense* may have spleens 20 times as large as normal (Mansfield and Bagasra 1978). In both cases, organ growth can be attributed primarily to marked hyperplasia of lymphoid elements, but the observed erythrophagocytosis (Greenblatt 1973; Goodwin 1974; Murray, P.K. et al. 1974a) is probably also a contributing factor.

Anemia has long been recognized as a common feature of the various pathogenic forms of trypanosomiasis as well as other parasitic diseases (Mulligan 1970; Woodruff et al. 1973; Goodwin 1970, 1974; Jennings 1976), and accumulating evidence strongly suggests that immunologic mechanisms promoting erythrophagocytosis are involved (see Jennings 1976; Kobayashi, Tizard, and Woo 1976; Jarvinen and Dalmasso 1977). Similarly, the anemia in rodent trypanosomiasis appears to be immunologically mediated, but because studies have been so limited, it is not clear which of the several possible mechanisms described by Jennings (1976) is operative. Most likely, immune complexes that promote erythrophagocytosis rather than intravascular hemolysis play a major role.

Human, animal, and experimental infections with the pathogenic trypanosomes have been reported to cause complement depletion (Ormerod 1970; Nagle et al. 1974; Greenwood and Whitttle 1976b; Kobayashi and Tizard 1976; Diffley 1978b), and there is evidence to suggest that immune complexes are also involved in this process (Nagle et al. 1974; Houba 1976; Neilsen and Sheppard 1977). Thus far, in studies of rodent trypanosomiasis, significant complement depletion has been reported only in *T. lewisi*-infected rats (Jarvinen and Dalmasso 1976), but here, too, immune complexes appear to play a major role. The thickened basement membranes reported in the glomerulonephritis of infected rodents, for example, most likely result from deposits of complement-containing immune complexes, which have been reported in experimental infections with pathogenic trypanosomes (Nagle et al. 1974; Houba 1976). It has been suggested, however, that other mechanisms of complement consumption, such as the release of complement-activating factors by the parasite, may also occur in trypanosomiasis (Nielsen and Sheppard 1977; Nielsen, Sheppard, Tizard et al. 1978). In this regard, Diffley (1978a, b) and Diffley and Honigberg (1978) have convincingly demonstrated that complement consumption can occur in the absence of an immune response. They found that in immunosuppressed rats infected with *T. congolense* or *T. rhodesiense*, complement levels were depressed and to a degree generally proportional to the number of circulating parasites. Moreover, the addition of trypanosomes from immunosuppressed hosts to fresh normal rat, human, and bovine sera caused immediate complement consumption in vitro, and C3 was detected on the surface of such parasites by immunofluorescence techniques. Similar direct activation of complement in vitro by suspensions of living *T. brucei* cells derived from lethally irradiated hosts as well as by isolated variant-specific surface antigen was recently reported by Musoke and Barbet (1977). In rodent trypanosomiasis, however, as mentioned earlier, there is as yet no evidence for direct activation of complement.

The significance of hypocomplementemia in trypanosomiasis is not fully understood, but its possible role in immunodepression, polyclonal stimulation, susceptibility to secondary infections, and evasion by the trypanosomes of the host's immune response has been discussed by others (Losos and Ikede 1972; Nielsen and Sheppard 1977; Nielsen, Sheppard, Holmes, et al. 1978; Diffley 1978; Cross 1978a). In rodent trypanosomiasis, there is evidence to suggest that depressed complement levels are directly related to

lowered resistance to secondary bacterial infection. In a study by Nielsen, Sheppard, Holmes, et al. (1978), it was found that if rats were inoculated with *Salmonella typhimurium* 1 week after infection with *T. lewisi*, a time when the hypocomplementemia is most severe, all the animals died within 5–12 hours of enteritis; in contrast, among control animals infected with bacteria alone, the death rate was only 10%.

Immunodepression is now a well recognized and studied feature of pathogenic forms of trypanosomiasis as well as other parasitic and infectious diseases (Goodwin 1970; Goodwin et al. 1972; Murray, P.K. et al. 1974a; Houba 1976; Clinton et al. 1975; Rowland and Kuhn 1978a, b; Ramos et al. 1978). Thus far, however, immunodepression in rodent trypanosomiasis has been described only in *T. musculi*-infected mice (Albright, Albright, and Dusanic 1977, 1978; Hazlett and Tizard 1978). On the basis of the limited data available, some interesting differences are apparent. In *T. musculi* infections, although there is an absolute increase in splenic T and B cells, the proportion of B to T cells declines to about half the normal value. In *T. rhodesiense*-infected mice there is also an absolute increase in B and T cells of the spleen, but the ratio of B to T cells increases to more than 6 times the normal (Mansfield and Bagasra 1978). The marked hyperplasia of plasma cells in experimental African trypanosomiasis has been noted by others (see Murray, P.K. et al. 1974a) and is no doubt the basis of the elevated IgM levels found in this disease, although the initiating factors are not completely understood (Clarkson 1976). Moreover, recent studies have provided evidence implicating suppressor T. cells and macrophages in the immunodepression of African trypanosomes (Corsini et al. 1977; Eardley and Jayawardena 1977); the trypanosomes do not appear to play a direct role (Eardley and Jayawardena 1977). In contrast, there is evidence that humoral factors of parasite origin have a direct immunodepressive effect in *T. musculi* infections (Albright, Albright, and Dusanic 1977, 1978). Nevertheless, these apparent differences in mechanisms do not alter the potentially important consequences of immunodepression, especially those related to secondary infections (see Murray, P.K. et al. 1974a).

It is apparent from comparisons of the abnormalities produced by the pathogenic and the rodent trypanosomes that there are many similarities. In rodent trypanosomiasis, however, even when pathologic changes approach in severity those produced by the pathogenic species, the duration of the changes is relatively short and there is a return to the normal state with few, if any, lasting effects. There is no doubt that the rapid immune response of the host attenuates the pathogenicity of the rodent trypanosomes and is the basis of the favourable host–parasite relationship.

Moreover, the central element of this response is ablastin, the unique reproduction-inhibiting antibody whose mode of action is still not completely understood (see D'Alesandro 1970, 1975; Giannini and D'Alesandro 1978). Present knowledge of rodent trypanosomiases has had no practical applications, but further studies leading to identification of the factors responsible for the comparatively benign nature of these diseases may well provide the key to control of their pathogenic counterparts.

Parasitemia and host susceptibility to African trypanosomiasis

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Abstract. Susceptibility to African trypanosomiasis would appear to be directly related to the capacity to limit, reduce or control parasitemia. This has been confirmed in both cattle and mice where breeds and strains show a range of susceptibility. Thus, the less susceptible Ndama breed of cattle show a superior capacity to control parasitemia than the more susceptible Zebu. Similarly, in inbred strains of mice there is a correlation between susceptibility and levels of parasitemia. There is evidence in mice that susceptibility to trypanosome infection is inherited as a dominant trait and is under complex genetic control; it seems likely that these differences in susceptibility are related to the host's immune response. One important fact to emerge is that the parasitemia kinetics are quite different between the bovine and the mouse. Even in the least susceptible strains of mice parasitemia is ongoing and death is inevitable, whereas cattle, especially the less susceptible Ndama breed, show ever decreasing waves of parasitemia until, as far as can be determined by existing parasitological techniques, the parasite is eliminated and the host in many cases recovers.

Antigenic variation is the biological phenomenon that dominates most considerations on African trypanosomiasis. It is the process whereby trypanosomes sequentially express a series of antigens on their surface. The immune response against each variant kills only the trypanosomes that possess that particular antigen and is invariably too late to affect that proportion of the population that has altered its antigenic identity. Parasitemia rises and falls in waves, with each new parasite population carrying a new surface coat antigen. As a result, it is usually assumed that parasitemia is ongoing until the death of the host; this can be a mistaken assumption.

Too often in such studies, little consideration is given to the accurate sequential quantification of parasitemia. It is important to realize that there is a whole range of variables, involving both the host and the trypanosome, that profoundly influence the parasitemia profile. These include the species, the breed, or strain of host, as well as the species, strain, and infective dose of the trypanosome. Some host species are completely refractile to infection with a particular trypanosome species or certain strains of a trypanosome species; this is termed innate resistance. On the other hand, within most host species in which trypanosome infections become established, there is considerable variation in susceptibility; this may be termed innate susceptibility. The results of our recent studies have shown that susceptibility is related to a host's capacity to limit the level of parasitemia along with the ability to control or even eliminate the trypanosomes and that this influences the entire outcome of the disease. Thus, in any studies on host susceptibility to trypanosomiasis it is essential that parasitemia be accurately evaluated.

It is now well recognized that certain breeds of domestic livestock and species of wildlife survive without chemotherapy in areas of tsetse fly challenge where other breeds and species cannot (reviewed by Murray, M., Morrison, Murray et al. in press). This phenomenon is known as trypanotolerance, although in immunologic terms this is a misnomer because trypanotolerant animals do in fact respond immunologically to the trypanosome. The lack of a vaccine and the limitations of the present methods of control, namely chemotherapy and tsetse control, have stimulated interest in the potential use of trypanotolerant livestock. However, there is a lack of data on most of the basic questions about trypanotolerance, namely to what extent is inheritance important and

how is it influenced by environmental factors, what are the mechanisms underlying increased resistance and, how productive are trypanotolerant breeds under various levels of challenge and in different management and ecological situations?

We have carried out a series of experiments on Ndama and Zebu breeds of cattle in an attempt to answer at least some of these questions. Particular attention has been focused on the Ndama breed because it has a relatively fixed phenotype and can be made productive; also published information has consistently indicated its greater resistance to trypanosomiasis over the Zebu (reviewed by Murray, M., Morrison, Murray et al. in press). In parallel, we have examined the susceptibility to trypanosomiasis of a range of inbred strains of mice (Morrison et al. 1978) and the underlying genetic basis of the susceptibility (Morrison and Murray submitted for publication). What has emerged clearly from these studies is that the susceptibility of a breed of cattle, of a strain of mouse, or of an individual within a breed or strain is directly related to the ability to limit the level of parasitemia and to the capacity to reduce or control parasitemia. In the bovine, anemia is the major disease-promoting factor (reviewed by Murray p. 121), and we have found that the severity of the anemia, and hence the disease, is directly related to the level of parasitemia, at least in the early phases of the disease, i.e., there is a strong correlation between the degree of susceptibility, the level of parasitemia, and the severity of anemia.

The accurate measurement of parasitemia, namely the prepatent period and the level and duration of parasitemia, is particularly a problem in the bovine where parasitemia is usually sporadic and scanty with levels that are difficult to detect by conventional quantitative techniques, e.g., hemocytometer. Visual scoring systems require considerable expertise to recognize a $\log_{10}$ difference in concentration, particularly at high parasitemia, e.g., 10^8 and 10^9 trypanosomes per ml, a difference that in the mouse can mean survival or death (Morrison et al. 1978). With this in mind, we now use a combination of the hemocytometer, which gives reliable results at levels of parasitemia above 10^5 trypanosomes per ml, and the blood buffy coat darkground/phase-contrast diagnostic technique adapted for scoring (Fig. 1). This technique has been described in detail elsewhere (Murray, Murray, and McIntyre 1977; Paris, Murray, and Agure in press). Although this method is only a semiquantitative estimate of the level of parasitemia, we have found that it gives consistent results.

Buffy coat DG/phase technique

Score	Trypanosomes observed using ×250 magnification	Trypanosome concentration
5+	>10/field	$>5 \times 10^5$/ml
4+	1 – 10/field	10^4– 5×10^5/ml
3+	0.1 – 0.5/fields	10^4/ml
2+	1 – 10/preparation	10^3 – 10^4/ml
1+	1/preparation	10^2 – 10^3/ml

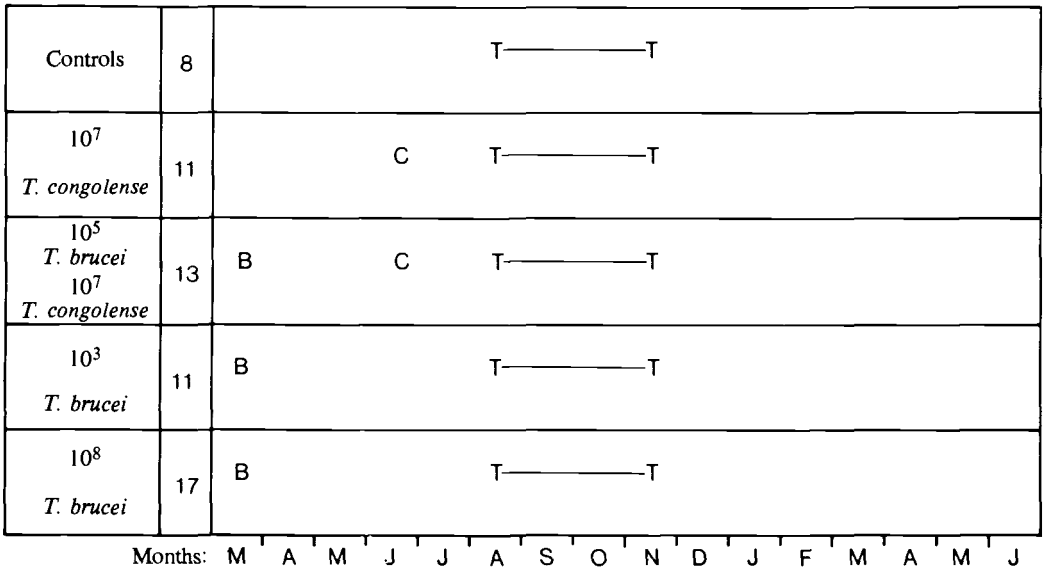
Fig. 1. *Scoring system (1–5+) based on the trypanosomes seen in a preparation or in a microscopic field (technique described by Murray, Murray, and McIntyre 1977).*

Ndama and Zebu

The protocol (Fig. 2) and preliminary results of one of our experiments have been detailed by Murray, P.K. et al. (in press a, in press b), and a report of the complete study is being prepared by Dr P.K. Murray. The cattle challenged with graded doses of *T. brucei* and or *T. congolense* were 3–4 years old and had not been previously exposed to trypanosomiasis, as judged by parasitologic and serologic examination and by the fact that they were obtained from tsetse-free areas. During the rains of August, the animals came under a natural *Glossina palpalis* tsetse fly challenge that lasted until November (Fig. 2).

Following inoculation, all Zebu and Ndama became infected and ill as judged by deterioration in physical condition and the development of anemia. During the course of the experiment, 12 infected Zebu and 15 infected Ndama were killed for histopathologic studies. Of the remaining 37 Ndama, none died, whereas 30 of the 40 Zebu died of trypanosomiasis, 21 during or after the fly challenge, and the other 9 because of the needle inoculation, 3 from *T. brucei*, 3 from *T. brucei* followed by *T. congolense*, and 3 from *T. congolense*. This study confirmed that a major difference in susceptibility to African trypanosomiasis exists between Ndama and Zebu.

Tables 1 and 2 show the parasitemia profile of infected Ndama and Zebu. What is immediately obvious is that the prepatent period was similar in both breeds of cattle but there was a significant difference in the level of the first peak of parasitemia, with Ndama having consistently lower levels of parasitemia. Another major feature of the parasitemia was that all the surviving Zebu and all Ndama were apparently able to eliminate trypano-



B = *T. brucei* challenge
C = *T. congolense* challenge
T—T = natural *Glossina palpalis* challenge

Fig. 2. *Experimental design used from March to June the following year.*

somes from their blood and tissues, in most cases several months before the termination of the experiment. There was also an indication that the duration of parasitemia was shorter in the Ndama but the picture was complicated by the fact that nearly all the Zebu and a few Ndama were reinfected during the *G. palpalis* challenge.

When challenged with *T. congolense*, the Ndama were able to control the first and subsequent waves of parasitemia to a much greater extent than the Zebu (Fig. 3). This reflected directly on the disease process as judged by the degree of anemia and the mortality in the Zebu. Similar results were obtained with *T. brucei*. Although the disease produced was less severe than that caused by *T. congolense*, we found that the isolate of *T. brucei* used was pathogenic for both Ndama and Zebu and was lethal for some Zebu (3). Following the disappearance of the parasites from the circulation, the majority of Ndama (25 of 37) and some Zebu (3 of 10) made a complete clinical recovery. The significance of these results is discussed elsewhere (Murray p. 121).

Because of the importance of the animal's potential to self-cure, we devoted considerable effort in the final 120 days of the experiment establishing whether the trypanosomes were completely eliminated, were too few to be detected, or perhaps were sequestered in some privileged site in the host. At weekly intervals, peripheral blood from the ear vein, as well as jugular blood samples, were examined for the presence of trypanosomes using the blood buffy coat darkground/phase contrast technique (Murray, Murray, and McIntyre 1977). At three separate monthly intervals, we inoculated two groups of rats intraperitoneally with 3 ml of jugular blood from every Ndama and Zebu. Tail blood from each rat was examined every 2 days for 2 months for the presence of trypanosomes. At the end of the study, we selected seven Ndama and three Zebu for necropsy. Tissue samples from choroid plexus, kidney, and heart were chopped up finely in RPMI 1640 medium prior to intraperitoneal inoculation into mice; five mice per tissue sample were used. Tail blood from all mice was examined for the presence of parasites at 2-day intervals for 30 days; subsequently, the mice were exsanguinated, the blood from each group pooled and subinoculated into further groups of two mice.

Between 90 and 120 days before termination of the study, we detected trypanosomes in one Zebu and three Ndama. Thereafter, all cattle remained negative. Trypanosomes were not found in any of the mice subinoculated with tissues from necropsied Ndama and Zebu.

The ability of both Ndama and Zebu to eliminate the trypanosome has been previously reported by several groups of workers studying small numbers of cattle (Stewart 1951; Chandler 1958; Desowitz

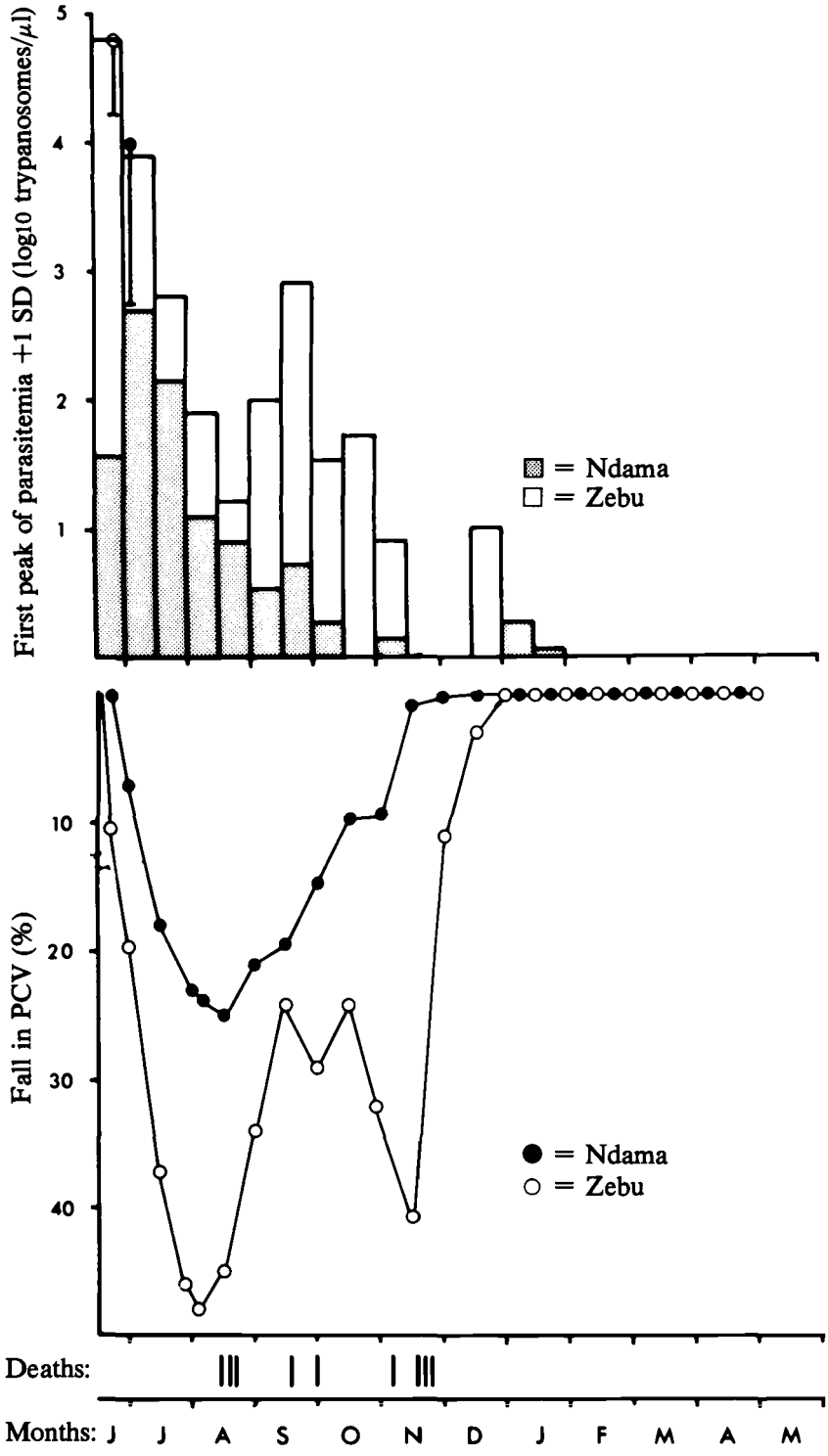


Fig. 3. Average parasitemia score, recorded during bimonthly intervals throughout an experiment with Ndama and Zebu inoculated with 10^7 T. congolense.

Table 1. Parasitemic profiles in Ndama and Zebu cattle inoculated with *T. brucei* and *T. congolense*.

Breed	Sample (no.)	Infection	Prepatent period (d)	First peak of parasitemia (Mean ± SD)
Ndama	17	10^8	2	3.8 ± 1.8
Zebu	17	*T. brucei*	2	5.0 ± 0.0
Ndama	11	10^3	11	1.4 ± 1.6
Zebu	11	*T. brucei*	11	2.5 ± 1.7
Ndama	13	10^7	6	3.5 ± 1.1
Zebu	13	*T. congolense*[a]	6	4.8 ± 0.6
Ndama	11	10^7	4	4.0 ± 1.3
Zebu	11	*T. congolense*	4	4.8 ± 0.6

[a]Infected with 10^5 *T. brucei* 56 days previously.

1959; Wilson 1971; Wilson and Cunningham 1971, 1972). Perhaps, because of the small number of cattle involved, the significance of this finding was never emphasized. We have confirmed these results using large numbers of cattle, and we suggest that this finding sheds a new light on the question of vaccination in the bovine.

The limitation of a trypanosome infection by the bovine preceded by a fluctuating, gradually diminishing level of parasitemia (Fig. 3) suggests the existence of an antigen, common to all variants of a trypanosome species, that has the ability to prime the host for a series of secondary and tertiary responses to newly appearing variants. Perhaps, the bovine has the ability to recognize this antigen whereas laboratory animal hosts such as the mouse have not. Or, it may be that although the potential number of variants is very high, under certain circumstances, as yet undefined, variants recur. This possibility is supported by evidence from Wilson and Cunningham (1972) and Nantulya et al. (submitted for publication) in serologic studies of *T. congolense*- and *T. brucei*-infected cattle. Another possible explanation is that in the bovine host there is a limited number of variants of any trypanosome isolate that show a high degree of virulence and that these variants are expressed during the early stages of infection (Van Meirvenne, Janssens, and Magnus 1975). In such a situation, cattle that survive this early phase of infection might then be able to eliminate less virulent variants and to recover.

We have found that in the bovine the parasitemic profile is influenced not only by the breed of cattle but by weight of inoculum, at least with *T. brucei*. Thus, when one compares the parasitemias of the groups of cattle receiving 10^3 and 10^8 *T. brucei*, the animals that received the lower challenge had a longer prepatent period, and significantly lower levels of parasitemia (Table 1). Consequently, the anemia was less severe in the animals receiving the low dose challenge. Similarly, at least in the Ndama, dose influenced the duration of the parasitemia (Table 2), the lower doses having a significantly shorter duration; it was impossible to tell whether the same was true for the Zebu because most of them became reinfected during the period of *G. palpalis* challenge.

In confirmation of the trypanotolerant nature of the Ndama breed, but reflecting the quantitative rather than the absolute nature of trypanotolerance, we found that Ndama receiving the highest chal-

Table 2. Duration of parasitemia in Ndama and surviving Zebu inoculated with *T. brucei* and *T. congolense*.

Breed	Sample (no.)	Infection	Duration of parasitaemia (d) AM ± SD	Range
Ndama[a]	12	10^8	105 ± 72	28–270
Zebu	5	*T. brucei*	218 ± 94	68–289
Ndama[a]	9	10^3	69 ± 58	15–187
Zebu	3	*T. brucei*	290 ± 11	280–302
Ndama[b]	9	10^7	155 ± 51	96–223
Zebu[c]	1	*T. congolense*	170	–
Ndama[b]	7	10^7	111 ± 56	34–206
Zebu	1	*T. congolense*	193	–

[a]Duration of study = 411 days (*Glossina palpalis* challenge commenced on day 155).
[b]Duration of study = 315 days (*Glossina palpalis* challenge commenced on day 59).
[c]Infected with 10^5 *T. brucei* 56 days previously.

lenge (10^8 *T. brucei*) developed a disease of the same magnitude as the Zebu that were given 10^3 *T. brucei*. This was true for the initial part of the experiment but was then complicated by the onset of the fly challenge.

Inbred Strains of Mice

We have found that inbred strains of mice show a range of susceptibility to infection with African trypanosomes (Morrison et al. 1978). Although this is true for *T. brucei* and *T. vivax*, the majority of our investigations have been carried out with *T. congolense*. At one end of the scale are the C57$\beta\ell$/6J and AKR strains, which are the least susceptible, and at the other extreme is the A/J strain. We judged susceptibility by levels of parasitemia and survival (Table 3). It must be emphasized that, in contrast to the bovine, the mice with *T. congolense* and *T. brucei* infections always died.

We found that reduced susceptibility was directly related to the capacity of the mice to limit the level of the first and subsequent peaks of parasitemia, and also to reduce the parasitemia between peaks. Some strains (C57$\beta\ell$/6) had the ability to do this over several waves of parasitemia; others (C3H) were able to control only the initial peaks; and some strains (A/J) failed completely to control parasitemia (Fig. 4). This was despite the fact that the prepatent period was similar in all the strains. The result was that the C57$\beta\ell$/6 strain survived longer than did the C3H, which in turn lived longer than the A/J. It would appear that the strains of mice that can limit their levels of parasitemia to less than 10^5 trypanosomes per μl show a much greater ability to control subsequent parasitemia and, as a result, to survive for long periods. When mice develop levels of parasitemia greater than 10^5 trypanosomes per μl, death usually follows. This difference underlines the need for accurate quantification of parasitemia as opposed to the approximations given by scoring systems especially at high parasite concentrations.

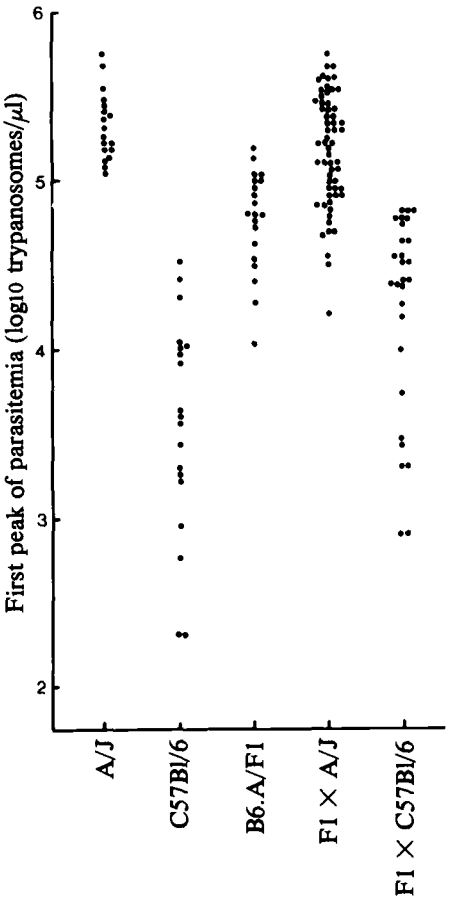


Fig. 4. *First parasitemic peaks of A/J, C57$\beta\ell$/6, F_1 hybrid, and backcross mice infected with* T. congolense *(Morrison and Murray in press).*

Table 3. Survival times for inbred strains of mice infected with *T. congolense*.

Mouse strain	Deaths (%) Days after inoculation 15	20	40	60	80	120	140	Mean survival time (d)
A/J	67.8	85.7	100	–	–	–	–	15.8
SWR/J	42.0	88.0	100	–	–	–	–	16.9
129/J	36.6	58.5	92.2	100	–	–	–	22.6
BALB/c/A	15.5	20.0	28.8	64.4	100	–	–	49.5
DBA/1J	0	0	62.2	100	–	–	–	36.3
C3H/HeJ	0	0	12.5	50.0	100	–	–	59.0
AKR/A	0	0	7.9	13.6	45.4	97.7	100	81.7
C57$\beta\ell$/6J	0	0	2.8	5.5	13.9	61.1	94.4	109

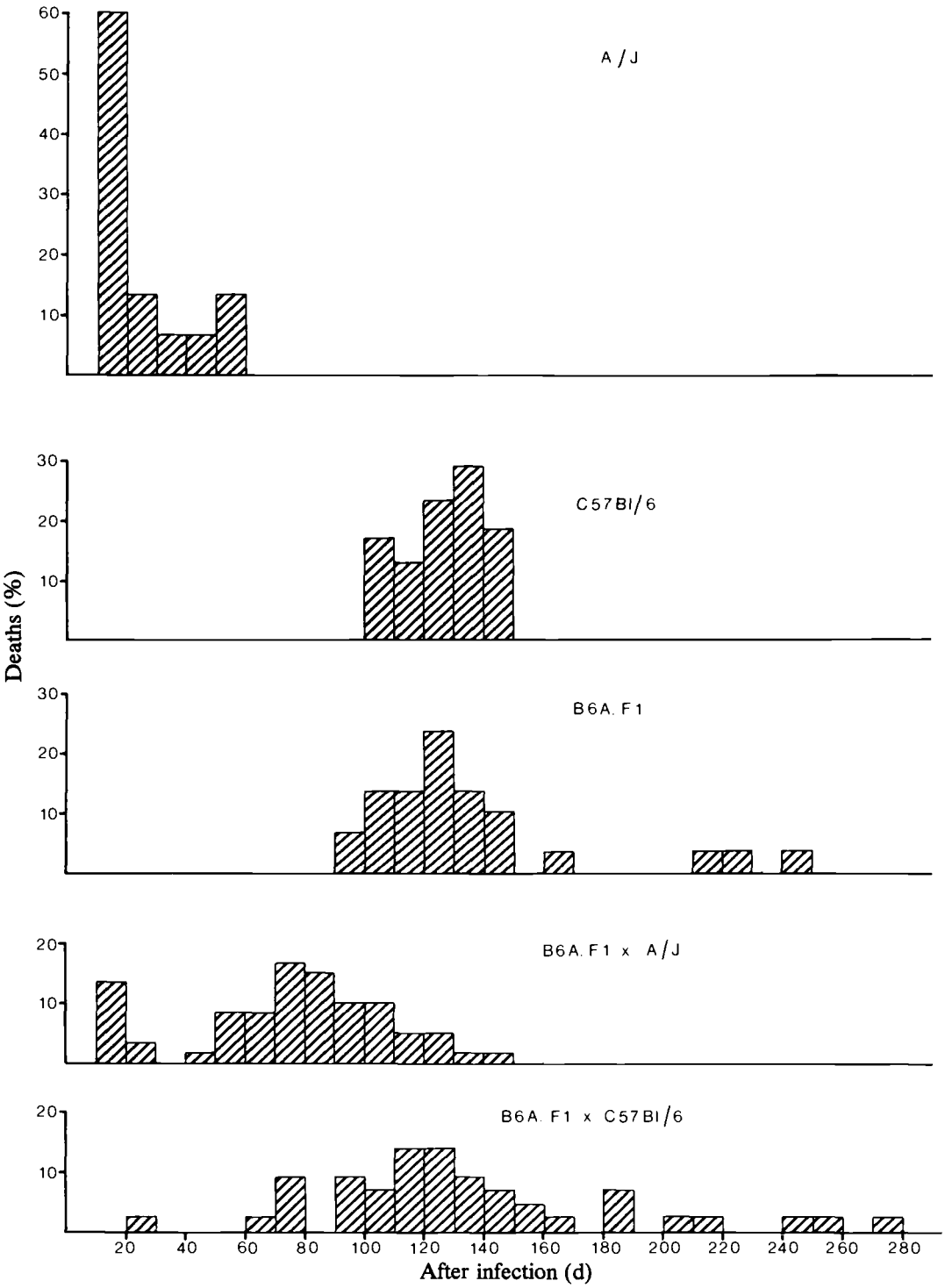


Fig. 5. *Percentage deaths at 10-day intervals in A/J, C57βl/6, F_1 hybrid, and backcross mice infected with* T. congolense *(Morrison and Murray in press).*

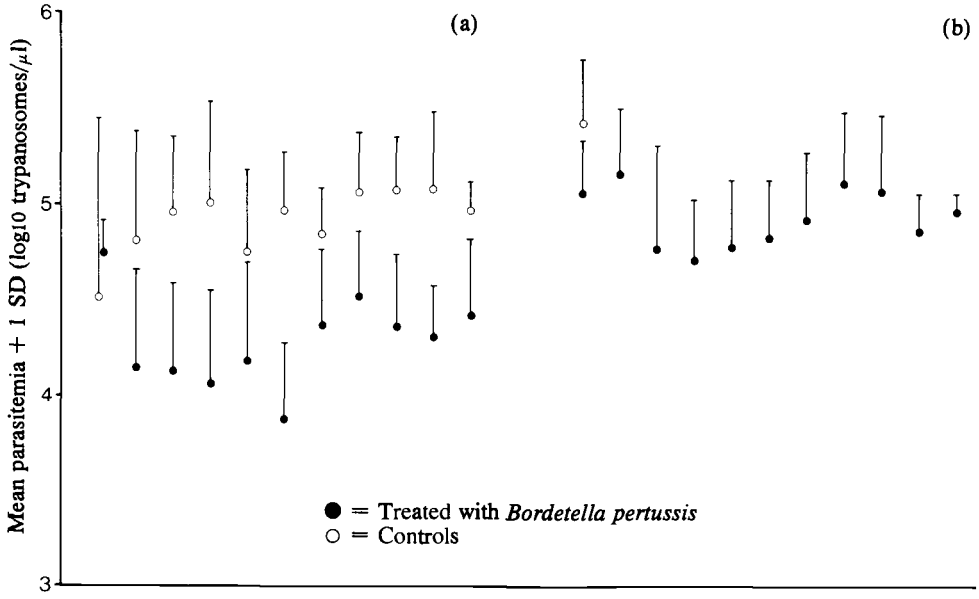


Fig. 6. *Peaks of parasitemia in* (a) *C57βℓ/6J and (b) A/J mice challenged with* T. congolense.

Our findings confirmed that the capacity to control parasitemia is under genetic control and is inherited as a dominant trait, although the degree of dominance depends on the parental combination (Morrison and Murray submitted for publication). For instance, F_1 hybrids (B6.A/F_1) between the highly susceptible A/J strain and the more resistant C57$\beta\ell$/6 showed survival times similar to the C57$\beta\ell$ parents (Fig. 5); this was reflected by their capacity to limit and reduce levels of parasitemia in a similar way but not quite to the same extent as the C57$\beta\ell$/6J parents (Fig. 4). Backcrosses of these F_1 hybrids with the C57$\beta\ell$/6 parents showed a similar pattern of parasitemia to the parents, although there was a greater scatter in survival times so that a few animals lived for longer than either of the parental strains (Fig. 5). The results of backcrossing the F_1 hybrids on to A/J parents suggested the possibility of a two gene control of susceptibility; of these backcrosses, approximately 25% showed a survival time similar to A/J, 25% were similar to the C57$\beta\ell$/6J and F_1, and the rest died in the intermediate period (Fig. 5). Furthermore, just under 25% maintained high levels of parasitemia similar to the A/J, whereas about 70% had a high initial peak but developed a distinct undulating pattern of parasitemia. However, the greater heterogeneity in survival of (B6.A/F_1 × C57$\beta\ell$/6) backcross compared with the C57$\beta\ell$/6 and B6.A/F_1 (Fig. 5) along with the less pronounced degree of dominance found in F_1 hybrid combinations of A/J and C57$\beta\ell$/6J with C3H/He (Morrison and Murray submitted for publication) suggests the involvement of additional genes.

In recent years, the susceptibility to a number of experimental infections in mice has been shown to be at least partially linked to H-2 haplotype (Lilly and Pincus 1973; McDevitt, Oldstone, and Pincus 1974). It has been suggested that H-2 may exert its influence through immune response (Ir) genes present in the I region of the H-2 complex. These genes are antigen specific, and they determine whether or not the mouse is able to mount a high antibody response to the antigen. However, our studies on the comparative susceptibility of congenic-resistant mice, i.e., mice with a genetic background differing only at the H-2 locus, have failed to demonstrate a major relationship between H-2 haplotypes and susceptibility; all showed similar patterns of parasitemia and survival. So far, we have carried out these experiments only on mice of the C57$\beta\ell$ genetic background, and it may be that the genes responsible for reduced susceptibility in this strain override any influence exerted by the H-2 haplotype.

Host Mechanisms in Control of Parasitemia

Although the basic mechanism responsible for controlling parasitemia is not clearly understood, there is evidence that it is related to a host response factor that is inherited as a dominant trait (Morrison and Murray submitted for publication). The finding

Table 4. Survival times of infected mice treated with *Bordetella pertussis*.

After challenge (d)	Surviving (%)			
	A/J[a]		C57βℓ/6J[a]	
	Control	*B.pertussis*	Control	*B.pertussis*
10	68	96	100	100
15	0	43	88	100
20	–	43	88	96
30	–	43	88	96
40	–	39	80	96
50	–	35	80	91
100	–	8	26	64
150	–	0	0	24
Mean survival time (d)	11.2 ± 1	26.4 ± 24.6[b]	74.5 ± 34.5	113.3 ± 47.8[b]

[a]25 mice per group.
[b]Significantly different from controls (arithmetic mean ± one standard deviation).

of a similar prepatent period between Ndama and Zebu cattle and between strains of mice of high and low susceptibility indicates that the initial replication rates are similar irrespective of susceptibility. Furthermore, dose titration studies showed that there was no difference in the infectivity of *T. congolense* for mice of high and low susceptibility (Morrison et al. 1978). These results suggest that the difference in levels of parasitemia between breeds of cattle and strains of mice may reflect differences in the nature or quality of the immune response to the trypanosome.

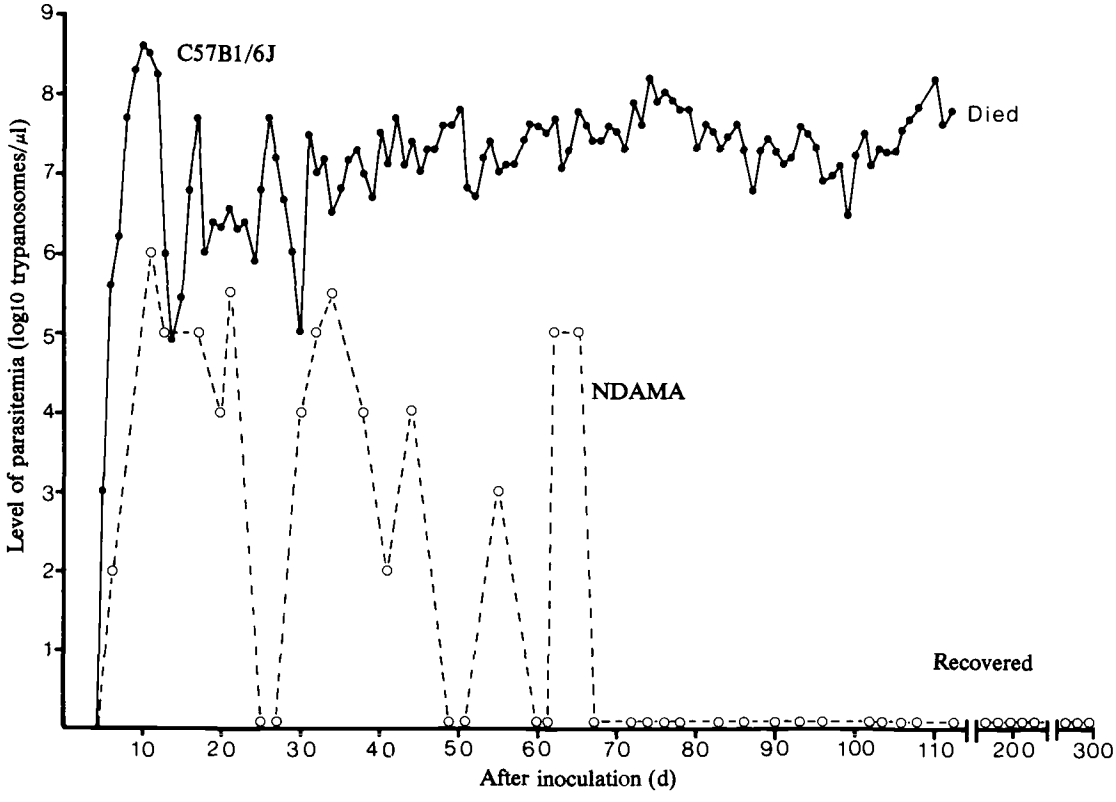


Fig. 7. *Levels of parasitemia found in a C57βℓ/6J mouse and a Ndama, which were infected with* T. congolense.

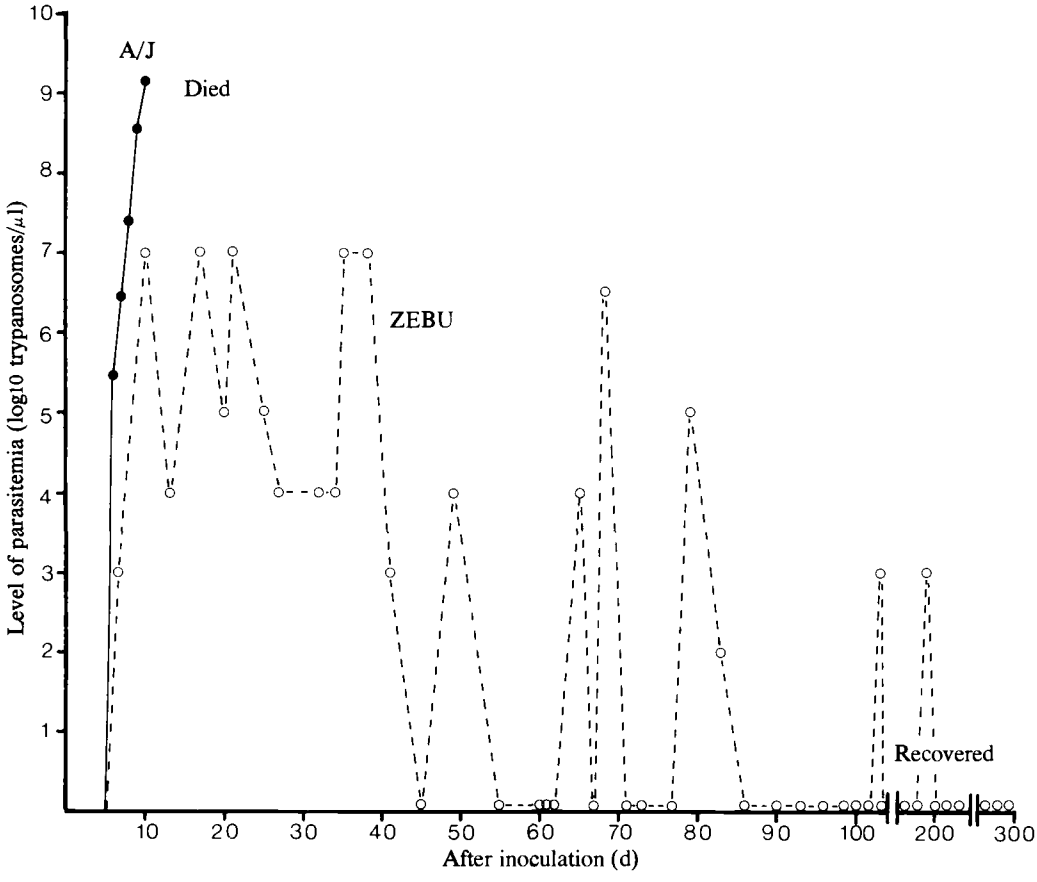


Fig. 8. Levels of parasitemia found in an A/J mouse and a Zebu, which were infected with T. congolense.

Evidence that this may be the case in cattle comes from the work of Desowitz (1959). He found that Ndama, with previous experience of trypanosomiasis, were able to control parasitemia and eliminate trypanosomes more rapidly than their Zebu counterparts following a renewed challenge. Employing an in vitro test that involved the use of sera from the challenged animals to inhibit trypanosome respiration, he demonstrated that the activity of Ndama sera was superior to that of Zebu sera. He concluded that the trypanotolerant nature of the Ndama was attributable to their capacity to mount a better secondary immune response. Unfortunately, in his studies, the trypanosomal antigenic history of the Ndama and Zebu was not precisely known. Although the results are indicative of a more effective immune response in the Ndama, they require confirmation. Similarly, using a serum neutralization test, Chandler (1958) stated without supplying details that the immune response of Zebu to the trypanosome was inferior to the Ndama.

Further evidence that reduced host susceptibility to trypanosomiasis is related to the capacity to control parasitemia as a result of a superior immune response comes from studies on the effect of immunostimulants on the susceptibility of mice to trypanosomiasis (Murray and Morrison submitted for publication). We have found that the administration of *Bordetella pertussis, Corynebacterium parvum*, or Bacillus Calmette-Guérin (BCG) prior to or on the day of challenge with *T. congolense* significantly delayed or reduced parasitemias (Fig. 6) and, as a result, increased survival times (Table 4). In this way, it was possible to alter the parasitemia and hence survival times of the highly susceptible A/J to values more akin to the less susceptible C57$\beta\ell$. It may be that this tactic can be practically employed with domestic livestock.

Also, we would suggest that in considerations of host susceptibility, attention be given to the relationship between parasitemia and nonspecific factors such as the extent and activity of the mononuclear phagocyte system as well as complement, properdin, and conglutinin reactivity.

Conclusion

Evidence has been presented to show that susceptibility to trypanosomiasis is related to the capacity to limit the level and, at least in the bovine, the duration of parasitemia. This ability is affected by host species, breed or strain, and possibly also by level of challenge. It has been established that susceptibility has a genetic basis and is inherited as a dominant trait, although it is almost certainly under polygenic control. It appears that the immune response plays a major role in controlling parasitemia but the evidence is not definitive. Although remarkable similarities were shown to exist between cattle and mice in relation to susceptibility to trypanosomiasis, there are considerable differences between the parasitemia that occurs in the mouse and that found in the bovine, and great care must be taken before extrapolating results between species. The level of parasitemia is considerably greater in the mouse and it is ongoing until death, whereas in the bovine there can be ever decreasing levels of parasitemia until apparent self-cure (Fig. 7 and 8). It is obvious that the course of infection in cattle is quite different from that in mice. Furthermore, the finding that large numbers of cattle are apparently able to limit a trypanosome infection must increase the hope that any future immunization procedure, although it may not be effective in mice, may have practical application in the bovine.

Immunity in the bovine to *T. congolense* induced by self-cure or chemotherapy

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Abstract. We tested the hypothesis that cattle acquired immunity to trypanosomiasis after self-cure or chemotherapeutic cure. Using Hereford cattle from trypanosome-free areas of Kenya, we intravenously infected 42 animals with *T. congolense* and monitored their progress until they self-cured, required diminazene aceturate (Berenil) treatment for survival, or died. Later, the surviving animals and a control group were challenged with a homologous strain of *T. congolense*. Our results during primary infection indicated that animals aged 4 months to 1 year have a strong resistance to the pathogenic effects of trypanosomiasis — 10 of 11 animals in that age group experienced self-cure, whereas all 20 animals more than 2 years old either died or required drug therapy to survive. After the second challenge, adult animals that had survived the first infection exhibited some immunity; they showed no clinical signs of disease from a third challenge.

Although many workers have attempted to induce immunity to trypanosomiasis under field conditions, their results have been contradictory. Reports of several investigators have shown no evidence of immunity in cattle maintained in endemic areas over long periods (Hornby 1941; Wilson, Paris, and Dar 1975). Other studies, however, claim that drug therapy induces a degree of protective immunity (Bevan 1928; van Saceghem 1938; Fiennes 1953; Soltys 1955; Smith 1958; Wilson et al. 1976). In some instances, these observations are difficult to interpret because of the nature of the drugs used to cure or suppress trypanosome infections.

It has also been postulated that young animals show an increased resistance to infection (reviewed by Fiennes 1970), possibly through transmission of an immune factor from the mother (Whiteside 1962). Other authors disagree and claim there is no evidence for naturally acquired immunity except in resistant breeds of cattle (Weitz 1970). No substantial laboratory investigations have been performed to resolve the conflicts concerning drug-induced or age resistance to trypanosomiasis. In this regard, we initiated a series of experiments to investigate the questions of natural and acquired immunity to trypanosomiasis in bovines.

Materials and Methods

Cattle, predominantly Hereford, were obtained from the veterinary department farm at Kabete or from other trypanosomiasis-free areas in Kenya. All animals were treated with phenamidine isethionate and oxytetracycline (Terramycin) to eradicate any latent babesia or anaplasma infections, dipped in an acaracide weekly and treated periodically with triclofenol piperazine (Ranizole) to limit helminthic infection.

We used the Trans Mara-I strain of *T. congolense*, which was isolated from an infected cow in the Trans Mara area near the Kenya-Tanzania border in 1966. The strain had been stored as a stabilate in dry ice and maintained in cattle by blood passage. We infected the cattle with trypanosomes obtained from infected bovines or mice. We enumerated the trypanosomes in a hemocytometer and diluted them with phosphate-buffered saline (pH 7.8) containing 5% glucose and 10% fetal calf serum. Cattle were infected intravenously with 1×10^4 *T. congolense*/227 kg body weight.

A second strain of *T. congolense* (Yoani-I) was used to test the specificity of immunity in animals immunized with the Trans Mara-I strain. This strain

Table 1. Effects of trypanosome dose on prepatent periods.

Dose (tryps/ 227 kg) (±2 SE)	Animals (no.)	Prepatent period (d) (±2 SE)
$3.0 (\pm 0.6) \times 10^8$	2	2.0 (±0.0)
$1.7 (\pm 0.5) \times 10^7$	2	3.0 (±0.0)
$7.0 (\pm 1.0) \times 10^6$	3	3.3 (±0.7)
$1.2 (\pm 0.2) \times 10^5$	9	4.6 (±0.5)
$1.1 (\pm 0.2) \times 10^4$	28	5.7 (±0.3)
$2.8 (\pm 0.1) \times 10^3$	2	7.0 (±0.0)

Table 2. Effect of age on *T. congolense* infections in cattle.

Age (y)	Animals (no.)	Median survival time (wk)[a]	Self-cures (%)
0.5–1	11	>78	10 (91)
1–2	11	25	2 (18)
2–3	11	12	0 (0)
3–4	5	6	0 (0)
4–5	2	7	0 (0)
5–6	2	8	0 (0)

[a]Based on time to treatment or day of death.

(Yoani-I) was isolated at Yoani, Kenya, in May 1977 from an infected dairy cow.

We reared *Glossina morsitans* by standard methods for fly-transmitted challenges. Newly emerged flies fed on an infected bovine donor 14 days. Thereafter, the flies fed for 5-day intervals on uninfected bovines until needed to induce infection.

We used diminazene aceturate (Berenil) to treat infected animals. The dose was 1.05 g active ingredient/300 kg body weight.

We obtained blood for smears from the tip of the tail 6 days a week and blood for hematological examinations from the jugular vein in disodium ethylenediaminetetracetic acid (EDTA) usually twice a week.

Parasitemias were estimated by counting the numbers of trypanosomes/100 leukocytes on thick blood smears and relating these values to the total leukocyte counts/mm^3. Thrombocyte numbers were determined by phase microscopy according to the method of Brecher and Cronkite (1950).

Results

Within the range of numbers of trypanosomes used in our experiments, we found no relationship between dose and animal survival time. Males and females did not differ in their response to infection or in survival time. The dose of trypanosomes, however, did affect the prepatent periods in experimental bovines (Table 1).

A marked resistance to infection was observed in animals aged 4 months to 1 year (Table 2). Ten of 11 animals 6 months to 1 year, and 2 of 11 animals aged 1–2 years survived infection. All animals more than 2 years old died or required treatment to survive.

When self-cured animals were challenged with the homologous strain of *T. congolense*, they did not develop detectable infections or any evidence of disease, whereas all control animals of comparable age required treatment to survive (Table 3).

Adult animals that required therapy to survive were also challenged with the homologous strain of *T. congolense* (Table 4). They had developed an appreciable immunity, and many of them self-cured the second infection. When the challenging infection was given about 2 years after treatment, the infections produced a chronic disease, and most animals had to be treated to survive. The infections

Table 3. Results of primary challenge of previously infected, self-cured animals.

	Initial infection						Primary challenge (1×10^4/227 kg)		
No.	Age	Sex[a]	Dose/ 227 kg	PP[b] (d)	Last patent parasitemia (wk-d)	Interval[c] (wk-d)	Age (y)	PP[b] (d)	Result (wk-d)[c]
1	0.5	M	2.8×10^3	8	54-4	25-0	2.0	NP	NDI
2	1.3	MC	1.0×10^4	5	61-1	31-6	3.0	NP	NDI
3	0.3	F	1.0×10^4	5	56-2	36-5	2.0	NP	NDI
4	0.5	M	2.9×10^3	6	30-5	48-5	2.0	NP	NDI
5	1.4	MC	1.0×10^4	5	38-2	54-5	3.1	NP	NDI
Average of 3 controls for primary challenge							3.1	4.7	T (9-3) [d]

[a]F = female; MC = Male castrated; M = male.
[b]PP = prepatent period; NP = not patent.
[c]Time between last patent parasitemia and challenge.
[d]Treated (time since challenge).

Table 4. Results of primary challenge of previously infected and treated cattle.

	Initial infection						Primary challenge (1 x 10^4/227 kg)		
No.	Age (y)	Sex[a]	Dose/ 227 kg	PP[b] (d)	Time to treatment (wk-d)	Interval[c] (wk-d)	Age (y)	PP[b] (d)	Result (wk-d)[d]
6	1.0	F	6.8×10^6	3	7-0	28-6	1.7	14	SC (17-0)
7	4.4	F	1.0×10^4	6	9-0	30-0	5.2	10	SC (11-5)
8	2.7	F	1.0×10^4	5	11-5	42-2	3.8	18	SC (15-5)
9	2.6	F	1.0×10^4	5	6-6	47-1	3.7	13	T (36-6)
10	1.9	F	1.3×10^5	5	28-0	71-4	3.9	14	SC (4-4)
11	1.6	MC	8.4×10^3	6	5-5	86-0	3.4	8	T (21-3)
12	1.9	MC	1.0×10^4	6	5-5	86-0	3.7	6	T (11-5)
13	2.3	MC	1.9×10^4	5	11-0	122-5	4.9	6	T (27-0)
14	3.4	F	1.3×10^4	5	5-1	128-4	6.0	6	SC (29-2)
Average of 8 controls for primary challenge							4.1	5.5	T (9-4)

[a] F = female; MC = male castrated.
[b] PP = prepatent period.
[c] Time between treatment and challenge.
[d] SC = self cure (time of last patent parasitemia after challenge); T = treated (time since challenge).

were less serious than those of controls, and treatment was required later than in the control group or during the primary infection. When animals were challenged a second time with a homologous infection, no detectable infections or clinical signs of disease were observed, whereas all controls developed parasitemia and needed treatment to survive (Table 5).

Animals immune to challenge with blood forms were also largely immune to tsetse challenge with the same strain (Table 6). Of 10 immune animals challenged by fly bite, 5 did not develop parasitemia or clinical evidence of disease. The others had limited periods of patent parasitemia and no serious clinical signs of disease (Table 7). All 10 immune animals survived. A severe infection developed in the eight controls, and seven required treatment. One control (aged 1.9 years) survived a severe infection.

No detectable immunity was observed when

Table 5. Results of the second challenge of bovines requiring treatment or self-curing after primary challenge.

Group	Animals (no.)	Interval[a] (m)	PP[b] (d)	Result[c]
Self-cure	3	5-30	NP	NDI
Treated	3	6-10	NP	NDI
Control	6	–	5-6	T (2.0 m)

[a] From last patent parasitemia or treatment.
[b] PP = prepatent period; NP = not patent.
[c] NDI = no detectable infection.

Table 6. Results of challenge of bovines immune to blood-form trypanosomes by tsetse fly bite (homologous strain).

Group	Animals (no.)	Patent (no.)	Median prepatent period (d)	Clinical signs	Survivors
Immune	10	5	>120	0	10
Control	8	8	10.5	8	1

Table 7. Clinical parameters of animals immune to blood form after challenge by tsetse flybite with the homologous strain of *T. congolense*.

Parameter	Group	Time after challenge (wk)								
		0	1	2	3	4	5	6	7	8
Packed cell volume (%)	Immune	31 (0.8)[a]	31 (1.0)	31 (1.0)	32 (0.7)	31 (0.9)	31 (0.9)	31 (0.9)	30 (0.9)	31 (0.9)
	Control	32 (0.5)	31 (0.6)	30 (0.6)	26 (0.8)	24 (0.7)	20 (0.5)	19 (0.3)	19 (0.7)	18 (0.5)
Thrombocytes ($\times 10^3/mm^3$)	Immune	466 (52)	432 (31)	410 (42)	415 (60)	398 (39)	415 (89)	464 (83)	369 (47)	333 (57)
	Control	533 (60)	608 (57)	258 (59)	120 (20)	130 (19)	196 (42)	151 (48)	168 (14)	187 (41)
Leukocytes ($\times 10^3/mm^3$)	Immune	13.7(1.2)	11.1(1.1)	12.7(1.4)	10.8(0.6)	10.6(0.6)	14.4(2.0)	12.6(1.4)	11.0(0.9)	11.8(0.6)
	Control	11.2(0.8)	10.5(0.7)	7.3(1.0)	6.9(1.4)	5.0(0.5)	5.6(0.6)	5.1(0.5)	5.2(0.9)	4.4(0.9)
Parasitemia (daily avg/mm^3)	Immune	0	0	147	470	117	137	299	0	524
	Control	0	0	4360	17163	3197	4039	7810	3781	3123
Animals patent/ animals remaining	Immune	0/10	0/10	3/10	2/10	2/10	2/10	2/10	0/10	3/10
	Control	0/8	0/8	8/8	8/8	8/8	8/8	7/7	6/6	6/6

[a] (1 standard error).

bovines, immune to the Trans Mara-I strain of *T. congolense*, were challenged with the Yoani-I strain, either by blood-induced or fly-induced challenge. Parasitemias and clinical evidence of disease in immune and control groups were undistinguishable, and all animals were treated during the 5th week after challenge, when packed cell volumes decreased to less than 20%.

Discussion

Our studies demonstrated an age resistance to *T. congolense* infection in bovines. Although young animals developed a relatively serious disease, almost all survived, whereas animals more than 2 years old invariably succumbed to infection. Although the mechanism(s) for the resistance is not known, it did not involve specific maternal antibody because mothers of our animals had never been infected. Our studies confirm and extend the observations of Fiennes (1970) whose results have not been widely accepted. Though Weitz (1970) asserted there was no evidence for an acquired protection in animals after recovery from the disease, we have shown that young surviving animals are resistant for extended periods to a challenge infection of the same strain by either syringe inoculation of blood forms or by tsetse fly bite (metacyclic forms).

Animals undergoing infection and cure were also resistant to challenge by the same strain. This protection was not premunitive, because the animals had been given curative therapy. The resistance correlated with the duration of infection, the time elapsing between treatment and challenge, and the number of infections to which the animals had been subjected. Our results support the field work of Wilson et al. (1976) who have shown convincing evidence that infected cattle develop immunity after treatment with diminazene aceturate (Berenil) in an endemic area. Effective levels of the drug persist in the blood of the bovine too short a time to complicate the studies. Trials in our laboratory (unpublished data) showed that diminazene aceturate (Berenil) (7 mg/kg) influenced infectivity for up to 12 days, the prepatent period up to 18 days but not later. These findings agree with previously published work (Cunningham et al. 1964).

Self-cure or cure by chemotherapy both largely protected animals against challenge by tsetse fly carrying the same strain of trypanosome. This fact indicates that cyclical transmission did not produce populations of parasites possessing different variant antigens from those expressed during the blood-induced infections.

Acknowledgments

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Trypanosomiasis of game animals

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Abstract. Wild mammals are sometimes said to be "trypanotolerant," i.e., they harbour trypanosomes but do not develop the clinical signs of trypanosomiasis. Although there are many studies that confirm the presence of trypanosomes in the blood of game animals, there are few, if any, that examine the pathological effects. At Kabete, we at the Veterinary Research Laboratory have begun studies to monitor the pathogenesis of the trypanosomiasis in animals that are trypanotolerant. Our preliminary results indicate that the animals act as reservoirs for the trypanosomes but do not suffer any adverse effects. More complete information will be forthcoming from game animals that we have raised and kept trypanosome-free.

Wild animals are usually said to be resistant to trypanosomiasis but highly susceptible to infection. In other words, the host–parasite association has evolved naturally so that susceptible wild hosts usually are free of the normal signs of disease. Healthy wild mammals may serve as reservoirs for trypanosomes and yet be resistant to trypanosomiasis.

In this paper, "resistance" means resistant to disease, not infection. Trypanotolerant is another word for the same concept; therefore, some wild animals are trypanotolerant. They are probably not tolerant to trypanosome infection in the immunologic sense because antibodies are found in their blood (Drager and Mehlitz 1978).

There are many reports showing trypanosomes in the blood and tissues of healthy game animals (Burridge et al. 1970; Ashcroft, Burtt, and Fairbairn 1959; Lumsden 1962; Hoare 1972); some of the authors have demonstrated histopathological changes in game animals infected with trypanosomes (Losos and Gwamaka 1973). Laboratory and field studies to date do not prove that game animals are resistant to trypanosomiasis; in fact, a percentage, and at times a high percentage, become diseased but are eliminated by predators.

The literature on infection and pathogenicity of trypanosomes in game animals is quite limited, although there are many reports demonstrating trypanosomes in the blood and tissues of wild animals. McCulloch (1967) found trypanosomes of the *brucei* subgroup in the blood smears of two zebra (*Equus burcheli*). The zebra had lost weight, and McCulloch considered *T. brucei* to be a contributory factor. He found trypanosomes in the blood and brain smears of the zebra but did not employ any other diagnostic procedures, e.g., to detect bacteria viruses or tissue abnormalities, to rule out the presence of other pathogens.

Wild animals as trypanosome carriers have been investigated by various workers. Ashcroft, Burtt, and Fairbairn (1959) divided game animals into two groups on the basis of susceptibility to trypanosomiasis. Group one comprised animals usually killed by *T. rhodesiense* and *T. brucei* infections, including Thomson's gazelle, dik-dik, blue forest duiker, jackal, fox, ant bear, hyrax, serval, and monkey. Group two consisted of animals usually resistant or "tolerant to infection," including the warthog, bush-pig, porcupine, and baboon. The authors were dealing with *T. rhodesiense* and *T. brucei*, and the results may have been different for strains of *T. vivax* and *T. congolense*, which are usually very pathogenic for cattle. While examining wild animals as a potential reservoir for *T. rhodesiense*, Geigy, Mwambu, and Kauffmann (1971) isolated 12 strains of the *T. brucei* subgroup: 2 from hyena, 5 from lion, 1 from warthog, 1 from waterbuck, and 3 from hartebeest. These strains were isolated from clinically healthy animals and their pathogenicity was not discussed.

There is some evidence that trypanosomes cause lesions in game animals and even kill them. For example, Losos and Gwamaka (1973) carried out a histological examination of wild animals described by Geigy, Mwambu, and Kauffmann (1971). Significant histological lesions were found in two impalas, one Thomson's gazelle, three Coke's

hartebeest, and in two lions. The lesions, which were attributed to trypanosomes, were myocarditis and meningoencephalitis and were consistent with the observations made in domestic animals (Losos and Ikede 1972).

Trypanosome infections in wild animals may be influenced by the distribution of the tsetse fly vectors. Weitz and Glasgow (1956) showed that tsetse flies preferentially feed on certain game species, avoiding hartebeest, zebra, or topi and, in the case of *G. morsitans, G. swynnertoni*, and *G. austeni*, seeking blood from *suidae*. Thus, pigs may have played an important role in maintaining a variety of tsetse flies in the area studied.

Game animals play a significant role in the epidemiology of human trypanosomiasis because they act as reservoirs for trypanosomes. Heisch, McMahon, and Manson-Bahr (1958) isolated *T. rhodesiense* from a naturally infected bushbuck, which on inoculation into humans produced clinical disease.

As in domestic animals, trypanosomes in wild animals may localize in tissues, and hematologic examination for parasites is not sufficient to rule out their presence (Lumsden 1962). On the other hand, finding trypanosomes in the blood is an indication of infection but not necessarily of disease.

Experimental Studies

In the last few years, personnel at the Wildlife Disease Section, Kabete, isolated trypanosomes from eland and began a study of pathogenicity (Karstad, Grootenhuis, and Drevemo 1974). My work is a continuation of their work. We have encountered one major problem: finding susceptible, trypanosome-free wild animals. To solve this problem, we have bred a few species in captivity and now have offspring of eland, buffalo, waterbuck, wildebeest, and bushbuck. Using a syringe (mechanical), we have transmitted trypanosomes from game animals to domestic animals and from free-living game animals to captive wild animals both in the field and in the laboratory. In the field, game animals were drug immobilized; blood was taken from their jugular or peripheral veins and inoculated into mice and goats to make isolates. Trypanosomes were detected using the standard trypanosome detection methods and were stored in liquid nitrogen for further investigation in the laboratory.

Two eland heifers born in captivity were inoculated with the sixth mouse-passage of a strain of *T. congolense* isolated from a captive eland in the Kiboko area. They developed parasitemia of plus two (+ 2) (up to 10 trypanosomes observed per field) 7 days post inoculation. The animals were under observation for a period of 90 days. During this period there were no significant changes in blood parameters, and their temperatures remained within normal limits. They eventually eliminated the trypanosomes from their bodies and are now clinically healthy. We did not determine the impact of the trypanosomes on their tissues, organs, etc. because, at the time, we had very few experimental animals and could not afford to sacrifice any of them. In future, we plan to examine the immunologic responses also.

Discussion and Conclusions

The experimental eland heifers were highly susceptible to trypanosome infection but did not seem to be affected by the disease. It is possible that the strain of *T. congolense* had become adapted to eland and was not pathogenic for them. It is also possible that the *T. congolense* lost its pathogenicity in the passages through mice (six passages); however, our observations are similar to those reported by Karstad, Grootenhuis, and Drevemo (1974) who inoculated *T. congolense* into captive eland. In that study, the eland did not show any adverse effects despite continuous parasitemia for 2 1/2 months.

Our field and experimental data on game animals have revealed that buffalo and giraffe are the main reservoirs of *T. congolense* at Kiboko. Captive eland in a tsetse-infested paddock were found to be infected with trypanosomes, but several free-living eland were not. Also, repeatedly, clinically healthy buffalo have exhibited *T. vivax* infections, isolates of which have been very pathogenic for cattle.

There are a number of problems that emerge in the study of game animal trypanosomiasis. The ideal situation would be to conduct this kind of study in game animals not subjected to confinement, as capture and confinement subject game animals to abnormal stresses. The drug immobilization and the stresses of captivity probably influence the development of disease in the animals, and it is hoped that breeding and raising them in a tsetse-free environment will provide more valid observations.

Although game animals have been shown to be reservoirs of trypanosomes pathogenic to our livestock, the pathogenicity of the trypanosomes to game animals themselves has not been sufficiently investigated. The challenge that faces immunologists at the moment is to analyze the immune responses of game animals and to extract information that can be used to save the lives of millions of domestic animals.

Discussion summary

F.E.G. Cox and G.A.M. Cross

The initiation of any trypanosome infection depends on the introduction of the organisms into the host. Insufficient attention is sometimes given to the quality and quantity of the inoculum. Techniques exist for estimating the numbers of viable parasites, and it is essential that the numbers are appropriate to the nature and size of the host, the natural infection, and the requirements of the experiment. In general, the number of trypanosomes correlates with the prepatent period, but the intensity and duration of the ensuing infection are markedly affected by genetic and environmental factors. Counts of parasites in the bloodstream are usually the basis for estimating parasite burden but may be inappropriate, particularly for *T. brucei* infections. The technique generally used for the detection of parasites in the bloodstream may miss low level infections and it is essential that the techniques used should be appropriate to the aims of the experiment and to the conclusion to be drawn from it. The peritoneal route of infection is frequently used for convenience but the natural infection is through the skin where some multiplication may occur and this may be associated with local reactions.

When rabbits and calves are infected with *T. congolense* through the bite of infected *Glossina morsitans*, parasites remain and appear to multiply in a chancre at the site of the bite. Such chancres have not been recorded from cattle; several hundred skinned animals have been examined without any indication of such lesions. In goats, on the other hand, bites from tsetse flies infected with *T. congolense*, *T. brucei*, or *T. vivax* produce very distinct chancres within 2–3 hours of the bite. *T. vivax* lesion is very inflamed and the trypanosomes disperse subcutaneously from the bite. Uninfected tsetse produce no lesions. It is not clear whether or not these local reactions can be correlated with skin thickness or whether or not they occur after every infective bite. There is evidence to suggest that the "tissue forms" are morphologically different from the metacyclic and bloodstream forms.

Susceptibility to Infection

The patterns of trypanosome infections are under genetic control in mice and are probably also under genetic control in cattle and other animals. In particular, Ndama cattle are less susceptible than Zebu, but nothing is known about the genetic diversity within these breeds. Differences may occur within one breed of animals that have bred for many years in either endemic or trypanosome-free areas. The small Zebu cattle around Lake Victoria appear to survive well despite heavy and continual challenge. Despite the fact that almost all Ndama have hemoglobin A, there is no obvious correlation between susceptibility and a particular hemoglobin type. In human sleeping sickness it has been shown that hemoglobin type is not important.

Environmental factors are also probably important in trypanosomiasis, especially in relation to the effort an animal has to make to obtain sufficient food and water, a problem that is not normally faced by animals maintained under experimental conditions.

Although the prevalence of trypanosomes in wild animals has been studied, trypanosome infections in them have not been critically evaluated. From the evidence available, it seems that some wild animals, although infected, are not seriously affected by trypanosomes, and this is as true for animals bred and infected in captivity as it is for those living in the wild. This suggests that in some species the innate resistance exceeds that seen in so called trypanotolerant cattle. The implication of this situation in relation to the domestication of wild animals deserves serious consideration.

Is the anemia in bovine trypanosomiasis caused by immunologic mechanisms?

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Abstract. Red blood cells of cattle infected with *T. vivax* and *T. congolense* were tested by the direct antiglobulin test with rabbit antisera against bovine IgM, IgG, IgG1, IgG2, and C3. Sensitization of erythrocytes with IgM, IgG1, IgG2, and C3 was demonstrated in a few blood samples only. It was concluded that attachment of immune complexes to erythrocytes contributes to the anemia in trypanosomiasis. More experimental evidence, however, is required to assess the extent of involvement of immunologic mechanisms.

Anemia is a common feature of trypanosomiasis (Woodruff 1973; Losos and Ikede 1972; Maxie, Losos, and Tabel 1976). It appears to be predominantly hemolytic associated with decreased life span of erythrocytes (Mamo and Holmes 1975; Jennings 1976; Valli and Forsberg 1978) and extensive erythrophagocytosis (MacKenzie and Cruikshank 1973; Jennings et al. 1974; Murray 1974; Murray, M. et al. 1974; Brown and Losos 1977; Valli and Forsberg 1978). Its cause is not entirely clear. Disintegrating trypanosomes may release a toxin that is hemolytic for washed erythrocytes (Huan et al. 1975; Tizard et al. 1977; Tizard et al. 1978a,b) but is not active in the presence of serum (Tizard et al. 1978a). Or the anemia may be associated with immunologic mechanisms. For instance, in severe infections, the onset of anemia is related to the decline of the first wave of parasitemia and the rise of serum antibody (Assoku 1972; Balber 1974; Kobayashi, Tizard, and Woo 1976; MacKenzie et al. 1978). In addition, treatment with immunosuppressive drugs increases *T. brucei* parasitemia in mice but delays the onset and reduces the degree of anemia (Balber 1974). This indicates a relationship to the antibody response. There is one report of the presence of autoantibodies (cold agglutinins) to red blood cells in human trypanosomiasis (Yorke 1911). No other reports, however, support the potential involvement of an autoimmune mechanism. It has been suggested that the anemia is due to sensitization of erythrocytes with immune complexes resulting in erythrophagocytosis (Assoku 1972; Woo and Kobayashi 1975; Kobayashi, Tizard, and Woo 1976). Evidence for this suggestion comes from reports of positive Coombs tests with antisera against euglobulins (Zoutendyk and Gear 1951; Kobayashi, Tizard, and Woo 1976), antiserum against IgG (Maxie, Losos, and Tabel 1976), and antisera against C3 (Woodruff 1973; Kobayashi, Tizard, and Woo 1976). However, negative results with direct Coombs tests have also been reported in human and rabbit trypanosomiasis (Mackenzie and Boreham 1974a) and in trypanosomiasis of cattle (Mackenzie and Boreham 1974a; Maxie, Losos, and Tabel 1976) and mice (Assoku 1972; Ikede, Lule, and Terry 1977). Kobayashi, Tizard, and Woo (1976) reported that they were able to elute IgG1 from erythrocytes of cattle infected with *T. congolense*, and the IgG1 had antibody activity against *T. congolense*. The reported observations suggest that immunologic mechanisms play a role in the development of the anemia, but the exact nature and the extent of this involvement remain unclear. Recently, MacKenzie et al. (1978) demonstrated the presence of trypanosomal antigen on erythrocytes of sheep infected with *T. congolense*. They showed the presence of antigen on erythrocytes by the indirect fluorescent antibody test with hyperimmune antisera applied to whole blood.

They reported that repeated washing removed the antigen from the red blood cells, thus indicating that the antigen was not firmly attached to the erythrocytes. The authors suggested that the antigen concerned was an antigen released as a result of trypanolysis.

The present report was an attempt to reevaluate the degree of sensitization of erythrocytes by immunoglobulin and C3 in cattle infected with *T. congolense* or *T. vivax* by applying the direct Coombs test with antisera specific for bovine IgG, IgG1, IgG2, IgM, and C3.

Materials and Methods

Animals: 24 Boran steers, 9–10 months old, were divided at random into three groups of 8 animals. Eight steers were intravenously (i.v.) infected with 10^5 organisms of *T. congolense* EATRO 1800. Another 8 steers were infected i.v. with *T. vivax* EATRO 1721. In both cases the inoculum was blood collected from an infected calf at the first rise of parasitemia. The remaining 8 steers served as controls. Blood was collected daily, but Coombs tests were carried out only about every other day.

Direct Coombs Test: The direct antiglobulin tests were carried out with a rabbit antiserum against bovine IgG and four other rabbit antisera monospecific for bovine IgM, IgG1, IgG2, and C3. The antisera were tested for specificity by immunoelectrophoresis. All antisera were absorbed with sheep red blood cells and with a pool of red blood cells collected from all cattle used in the experiment. Tanned sheep red blood cells were sensitized with heat-inactivated bovine serum (Herbert 1973), and the agglutinating activity of the antisera was tested by using the sheep erythrocytes passively sensitized with bovine serum as indicator cells. This test served as a positive control in that it provided an estimation of the titre of the respective antisera.

All antiglobulin tests were carried out in a microtitre system (Cooke Engineering Co). The red blood cells were washed four times in a diluent of phosphate glucose saline pH 8.0 containing 0.5 ml heat-inactivated normal rabbit serum. The normal rabbit serum also was absorbed with a pool of red blood cells collected from all cattle used in the experiment. The final bovine erythrocyte suspension was made up to a 2% suspension in diluent. Antisera were titrated in doubling dilutions in diluent in 0.025 ml amounts in plastic microtitre plates, using the automatic micropipetter; 0.025 ml of 2% erythrocyte suspension was added to each of the antiglobulin dilutions, thoroughly mixed, covered with tape, and incubated at 37 °C for 1 hour. Then the plates were transferred to a refrigerator and were read the next morning.

Results

When tested against a 2% suspension of sheep red blood cells coated with bovine serum, the antisera had the following antibody titres: rabbit anti-IgG (RA-IgG) 1:1024, rabbit anti-IgM (RA-IgM) 1:1024, rabbit anti-IgG1 (RA-IgG1) 1:256, rabbit anti-IgG2 (RA-IgG2) 1:256, and rabbit anti-C3 (RA-C3) 1:1024.

Two types of agglutination patterns were observed with bovine erythrocytes. Type I occurred at low antiserum dilutions (1:2, 1:4) and appeared to involve most erythrocytes. It occurred with controls as well as with erythrocytes from infected animals, although it occurred more often with infected samples. This type was considered nonspecific and will be discussed below. Type II occurred at high antiserum dilutions (at 1:32 or higher). Only a part of the whole cell population seemed to be involved because most of the cells sedimented in a good round button, but the wall of the bottom of the well was covered with a thin layer of agglutinated cells. Macroscopically, it looked like a red haze and could, if superficially examined, be mistaken for hemolysis. This type of agglutination pattern was mostly associated with a negative prozone. It was never observed with control samples. It had titres within the range of the positive controls and thus was considered specific.

The presence of IgM, IgG1, IgG2, and C3 was demonstrated on erythrocytes of blood collected from cattle infected with *T. vivax* or *T. congolense* (Table 1). The incidence was very low: only 19 positive samples of 382 tested. No positive result was obtained with blood collected before 25 days post infection, although the packed cell volume already started to decline 9 days post infection. No positive tests were observed with antiserum against IgG — a finding that appears to be at variance with the positive results obtained with monospecific antisera to IgM, IgG1, and IgG2. Erythrocytes of three blood samples of one steer infected with *T. congolense* and of one blood sample collected from another steer infected with *T. congolense* showed agglutination.

Discussion

Carrying out antiglobulin tests on blood of cattle infected with trypanosomes, one has to keep in mind that the erythrocyte suspension itself contains

Table 1. Direct antiglobulin test on bovine erythrocytes.[a]

	Cattle (no.)	Samples (no.)	Samples positive, tested with antisera against: IgG	IgM	IgG1	IgG2	C3
Controls	8	208	0	0	0	0	0
T. congolense-infected	8	174	0	3	3	2	1
T. vivax-infected	8	204	0	6	2	1	1

[a] We conducted tests on 24 days between 2 days preinfection and 47 days post infection.

trypanosomes. In previous trials we experienced a problem of hemolysis during washing of blood collected from *T. congolense*-infected cattle. We may have lost a cell population that was altered. The incorporation of 0.5% normal rabbit serum in the diluent prevented hemolysis. As to the described first type of agglutination pattern, one has to consider sources of errors, i.e., contamination of diluent or antiserum due to frequent use and extended storage in the refrigerator. We became aware of this problem and stored only small amounts of the reagents in the refrigerator so that they were used up within a few days. When the antisera were initially absorbed with bovine erythrocytes, absorption was done with erythrocytes of normal blood. Maybe potential antibodies to young red blood cells were not sufficiently absorbed out. Thus, low titre agglutination may also have been due to antibodies to antigens on young red blood cells. Since the antibody titres to bovine serum proteins in the antisera used were demonstrated to be much higher in the positive control, it is inconceivable that the low titre agglutinations were due to antibodies to bovine immunoglobulins or C3.

We suggest that the described second type of agglutination was specific, as titres were within the range of positive controls. The negative results with rabbit anti-IgG were at variance with positive results obtained with rabbit antisera monospecific for IgG1 and IgG2. We have no ready explanation for this paradox but suggest that the relatively high titre antiserum to IgG did not produce agglutination because of negative prozones, i.e., antibody excess. Further titration might have produced positive results. Titration beyond dilutions of 1:2048, however, were not carried out.

Because only a part of the whole cell population was agglutinated, we think that only a proportion of the cell population was sensitized. One can argue that the antiglobulin test detects only the tip of an iceberg of an ongoing process. The incidence of sensitization of erythrocytes with immunoglobulins or C3 was demonstrated to be so low that it makes the test inconclusive. The results of the antiglobulin tests did not answer the question whether immunologic mechanisms do or do not play a major role in the anemia.

It has been shown that erythrocytes can be coated with trypanosomal material in vitro (Dodin and Fromentin 1963; Herbert and Inglis 1973: Woo and Soltys 1972; Woo and Kobayashi 1975). The few cases of autoagglutination reported in this study are interpreted to be due to alteration of red cells by material of disintegrated *T. congolense* organisms. We suggest that coating of erythrocytes by cellular material of trypanosomes after disintegration of the organisms in vivo is itself sufficient to alter the surface of erythrocytes and cause adherence to macrophages and subsequent erythrophagocytosis. This interpretation is in accordance with histologically observed erythrophagocytosis (Assoku 1972; MacKenzie and Cruikshank 1973; Mackenzie and Boreham 1974a; Jennings et al. 1974; Murray 1974; Murray, M. et al. 1974), the observed association of onset of anemia with the appearance of serum antibody to surface antigen (Assoku 1972; Balber 1974; Kobayashi, Tizard, and Woo 1976), and the demonstration of trypanosomal antigen on erythrocytes of infected animals (MacKenzie et al. 1978). Experiments designed to test the validity of the above interpretation could be carried out in vitro.

Complement in experimental trypanosomiasis

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Abstract. To assess the role of serum complement in trypanosomiasis, we performed a time-course study for 2 months using experimentally infected (with *T. congolense*) and control calves. Total hemolytic complement levels and complement component C1, C1q, and C3 levels were found to be depressed within 3 weeks of infection. Serum properdin levels fluctuated reflecting the cyclical changes in parasite counts of individual animals, whereas serum complement component C8 levels were not affected.

Serum protein turnover was also compared in infected and control calves. In all the infected animals, serum protein catabolism was elevated.

The continuous depression of some of the complement components indicates that mechanisms other than immune complex activation take place. Studies on trypanosome lysates have revealed that some of the constituents have the capacity to activate complement to a remarkable extent. At least two components are capable of activating complement. One component is a protease; it only accounts for a fraction of the total activating capacity of the lysate and was not studied in detail. The second component is of unknown chemical structure but possibly is a glycolipid; it was isolated from various trypanosome species. The physiochemical properties of the component and its effect in vivo have been studied.

Complement is an intricate multimolecular enzyme system found in the fluid phase of blood and other body fluids. It is considered a nonspecific destroyer of foreign antigen usually made specific by antibody binding to the antigen or activated directly via a number of pathways. Complement has basically two distinct biological functions: activation and cellular destruction; it plays an important role in an animal's natural defence against foreign antigens. Commonly, any tampering with the complement system in an animal significantly reduces the animal's ability to defend itself against pathogens as well as causing other important body functions to be impaired.

Complement activation in trypanosome-infected animals is well established (Kierszenbaum and Weinman 1977; Jarvinen and Dalmasso 1976; Kobayashi and Tizard 1976; Nagle et al. 1974). However, the pathway and mechanisms of activation are unclear and in the literature conflict. Nagle et al. (1974) clearly showed that monkeys experimentally infected with *T. rhodesiense* were hypocomplementemic, had decreased serum complement component C3 levels, and had progressive glomerulonephritis. Kobayashi and Tizard (1976) noted that anemia in calves experimentally infected with *T. congolense* was associated with decreases in parasitemia, appearance of complement-fixing antibody and depressed serum C3 levels. Both of these reports suggest complement is activated by immune complexes. Greenwood and Whittle (1976 b) reported evidence suggesting activation of both complement pathways (the classical and the alternate pathways) in the absence of any clinical evidence of immune complex disease in patients with Gambian sleeping sickness. Further, Jarvinen and Dalmasso (1976) concluded that *T. lewisi* infection of rats resulted in low grade activation of the alternate pathway with the main activation being that of the classical pathway. The apparent discrepancies may be the result of the different species of animals or trypanosomes but further investigations are needed to elucidate them.

Materials and Methods

We critically examined complement systems of calves during experimental *T. congolense* infec-

tion. We obtained sera three times every 2 days for 2 months from three infected and two control calves. We stored all samples at −70 °C and assayed them at the end of the experiments to avoid technical error. Total hemolytic serum complement levels were measured by the method of Barta and Barta (1972), and CH_{50} units were calculated by the von Krogh equation as described by Kabat and Mayer (1961) (Fig. 1).

We measured serum complement components C1, C1q, C3, and C8 by radial immunodiffusion (Mancini, Carbonera, and Heremans 1965).

We prepared antisera in rabbits using components purified by techniques similar to those employed in the preparation of human components. All component antisera were tested for monospecificity by standard immunologic techniques and were found to yield single immune precipitate rings in radial immunodiffusion. We used whole bovine sera as well as their original antigens upon absorption to remove contaminating antibody. The levels of complement components of the experimental animals were assayed.

As we were unable to visualize immune precipitate rings to measure properdin levels in the calf sera, we used a modification of radial immunodiffusion (Nielsen 1978). Briefly, this technique made use of radiolabeled antiproperdin serum to precipitate properdin in an agarose matrix. The areas of agarose in which the immune complexes formed were excised and enumerated for retained radioactivity.

To investigate catabolic rates, we obtained ammonium sulfate precipitates of homologous serum, radiolabeling them with ^{125}I. The serum was taken from three controls and three animals, which were later infected with *T. congolense*. The radioactive globulins were then injected intravenously into each donor animal after the infected group had reached their first parasitemic peak. Blood samples were taken immediately after injection and at regular intervals for the next 4 days. We tested radioactivity of 1.00 ml of serum from each bleeding from each animal to ascertain total globulin decay and performed reversed radioimmunodiffusion assays to calculate the half-life of IgM, IgG1, IgG2, IgA, IgE, C1, C1q, C3, C8, and properdin. We also measured the immunoglobulin, C1, and C3 levels in nasal washings.

We then used rat cultured *T. congolense* and *T. lewisi* to test for complement activation. *T. congolense* organisms freshly harvested from rat blood and purified by the method of Lanham and Godfrey (1970) were counted and aliquots of 2×10^8 microorganisms were incubated with 2 CH_{50} units of bovine complement. At 15-minute intervals, trypanosome−complement mixture supernates were tested for residual hemolytic complement activity.

To assess the adverse effects of secondary infections on hosts parasitized with trypanosomes, we decomplemented rats with the complement-activating substance of trypanosomes and then infected them with *T. lewisi* or *Salmonella typhimurium*. We also included rats that were decomplemented with cobra venom factor and then infected. The experimental plan is outlined in Table 1.

Results

Determining lysis due to complement, we found all the calves had approximately 14 CH_{50} units/ml of serum initially (Fig. 1). The control calves

Table 1. Injection schedule for rats included in decomplementation studies.

Group	Animals (no.)	*T. lewisi*	CoF[a] (units)	CAF-T[b] (mg)	*S. typhimurium*
1	10	10^9	80	–	–
2	10	10^9	–	–	10^9
3	10	10^9	–	–	–
4	10	–	80	–	10^9
5	10	–	80	–	–
6	10	–	–	–	10^9
7	10	–	–	–	–
8	6	–	–	1	–
9	6	–	–	1	10^9
10	6	10^9	–	1	–

[a] Cobra venom factor – injected in three equal amounts 24 hours prior to *T. lewisi* or *S. typhimurium* injection.

[b] Complement activating factor of *T. lewisi* injected in three equal amounts prior to injection with *T. lewisi* or *S. typhimurium*.

Source: Nielsen, Sheppard, Tizard, et al. (in press).

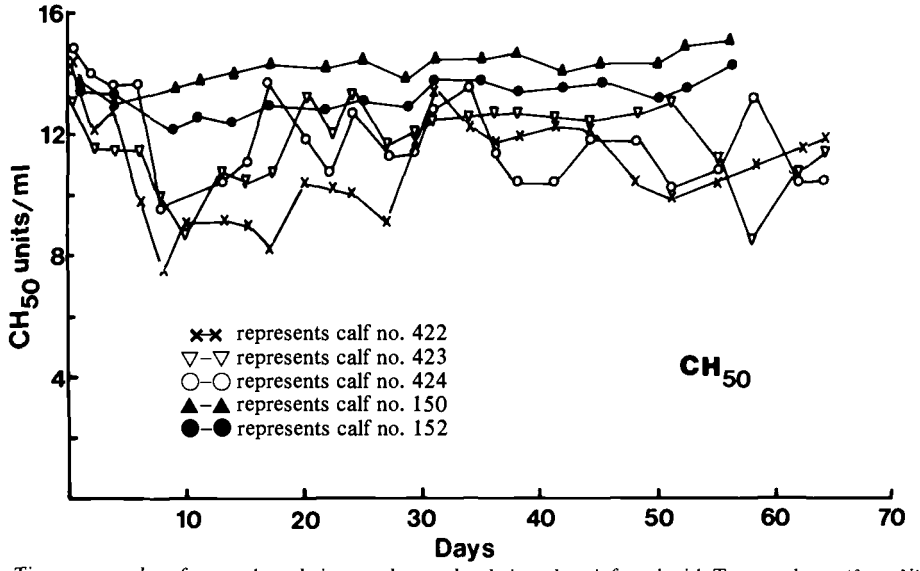

Fig. 1. Time-course plot of serum hemolytic complement levels in calves infected with T. congolense *(from Nielsen, Sheppard, Holmes et al. in press).*

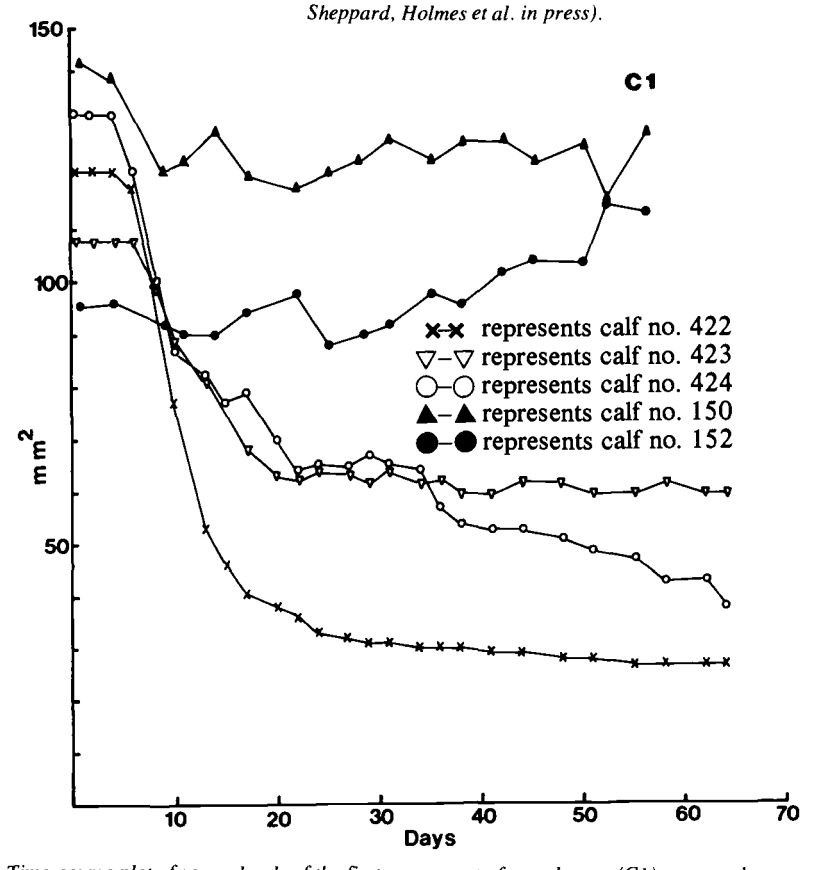

Fig. 2. Time-course plot of serum levels of the first component of complement (C1) measured as precipitin rings (mm^2) in radial immunodiffusion assays in calves infected with T. congolense *(from Nielsen, Sheppard, Holmes et al. in press).*

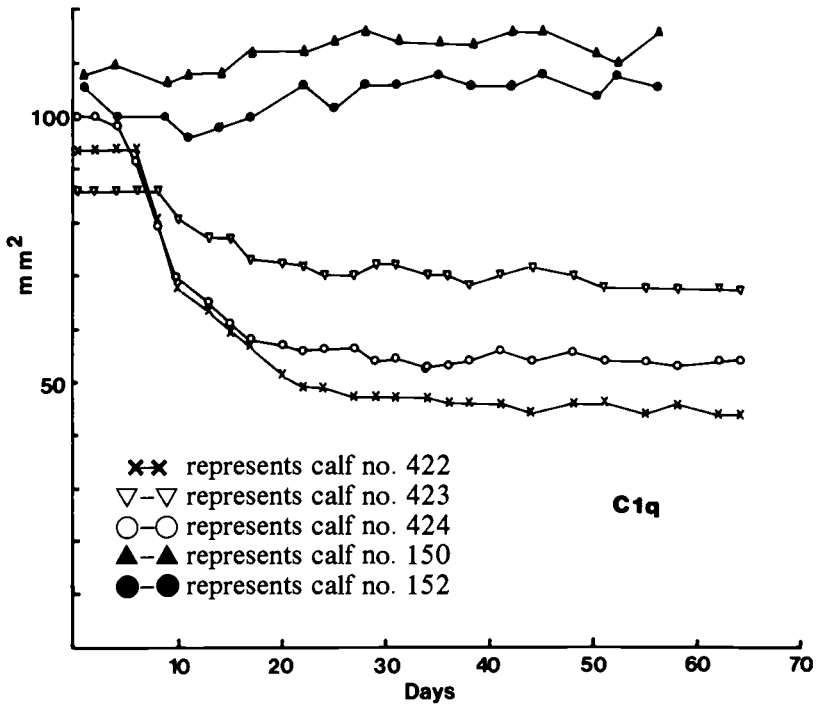

Fig. 3. *Time-course plot of serum levels of C1q subcomponent of the first component of complement measured as precipitin rings (mm^2) in radial immunodiffusion assays (from Nielsen, Sheppard, Holmes et al. in press).*

remained at this level, with minor fluctuation, throughout the study. The infected calves showed an initial decrease in CH_{50} levels about 8 days after infection. The initial increase in CH_{50} levels coincided with the first parasitemia peak but subsequently the fluctuations had no apparent relationship with the vascular parasite burden. It should be noted that the animal having the most severe parasitemia had the greatest decrease in complement levels.

The C1 levels of the five animals (Fig. 2) initially varied widely. The control calves' values fluctuated by as much as 10%, whereas the infected calves' values fluctuated up to 75%. The consumption of C1 began before infection became apparent, and C1 levels remained suppressed throughout. The levels of C1 appeared to depend on the overall blood parasitemia but were unaffected by the particular parasite counts. The levels of C1q and C3 reflected the results obtained in the C1 assays (Fig. 3 and 4.).

Infection with *T. congolense* did not have any influence on serum C8 levels.

The levels of properdin (Fig. 5) decreased and increased with the rise and fall of bloodstream parasites but were delayed 1–2 days after the changes in parasite levels.

Our results indicate that *T. congolense* infection has a profound effect on the serum complement system, particularly on the earlier components of the cascade and therefore perhaps on the total hemolytic complement levels. Although C1 is activated, C8 levels do not appear to be affected; therefore classical activation remains somewhat questionable but more than one mechanism of alternate pathway activation is evident.

The rates of disappearance of the ^{125}I-labeled globulin were considerably increased in infected animals. Unfortunately, the assay method we used was not sufficiently sensitive to obtain figures for the half-lives of C1q, C8, and properdin, but it revealed that serum C1 and C3 were removed from infected calves at much greater rates than from the control calves. On average, C1 and C3 respectively were catabolized 4.6 and 2.7 times as fast in infected calves. The immunoglobulins (Table 2) also showed abnormally rapid disappearance rates in infected calves.

Although IgM is generally elevated and IgG remains constant in calves infected with *T. congolense*, there is a very marked reduction (75–90%) in the serum concentrations of IgA and IgE because the catabolic rates of both these proteins are greatly increased (Nielsen, Sheppard, Holmes in press; Nielsen, Sheppard, Tizard, et al. in press). In considering this phenomenon, we felt

that a similar decline in the levels of secretory IgA would be found in hypogammaglobulinemia and could account for the increased susceptibility of infected animals to secondary infection (Losos and Ikede 1972). We, therefore, investigated the changes in the immunoglobulin contents of the nasal washings of *T. congolense*-infected calves (Table 3).

The serum IgM of both infected and normal calves was unaffected during the first 17 days of infection. However, the serum IgM rose in the infected animals and reached about 1.6 times the level in the normal animals by day 38. The highest serum IgM levels were reached in the animal (calf no. 19) with the lowest parasitemia. In contrast, the IgM levels in nasal washings did not differ markedly in the normal and the infected calves, although calf no. 19 had an inconstant elevation of its nasal IgM such that on day 38 it was approximately twice the normal level. When the IgM levels in each group were averaged and those for the group infected expressed as a percentage of the normal, a rise in nasal IgM levels was apparent, proportional to the rise observed in serum but so low that it was not statistically significant.

There was no significant difference between the normal and the infected animals with respect to nasal or serum IgG1 and IgG2 levels, although the values tended to be slightly lower in the infected calves.

Serum IgA levels in the infected calves were lower than in the controls, the average being about half that in controls. IgA levels in the nasal washings of infected animals, however, were not significantly lower than in the controls (Table 3).

Having established the depressed complement levels, their hypercatabolism, and their normal levels at mucosal surfaces as represented by the data from nasal washings, we investigated whether or not the trypanosome organism itself could activate complement. We found that a lag of about 3 1/2 hours preceded complement activation, and then microscopical observation revealed the near absence of living or intact trypanosomes in the

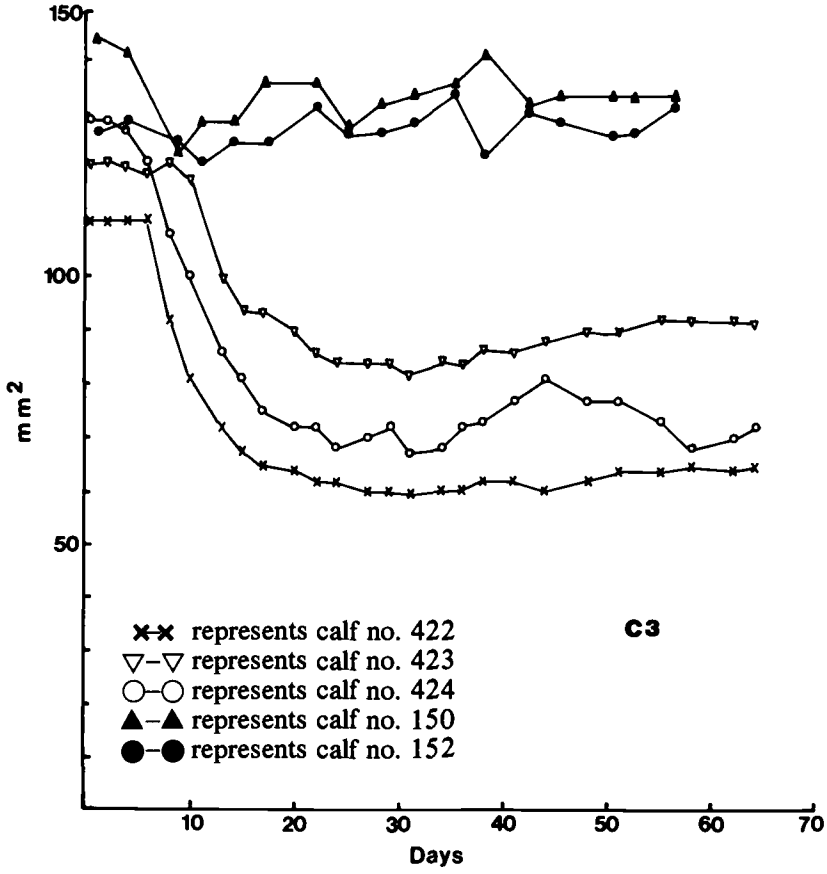

Fig. 4. Time-course plot of serum levels of the third component of complement (C3) measured as precipitin rings (mm²) in radial immunodiffusion assays (from Nielsen, Sheppard, Holmes et al. in press).

Table 2. Summary of the half-lives of serum immunoglobulin and complement components in control and *T. congolense*-infected calves.

Calf no.	Half-life (d)							
	Immunoglobulin	IgG1	IgG2	IgM	IgA	IgE	C1	C3
Trypanosome-infected								
115	1.46	1.23	1.33	0.58	0.75	0.92	1.29	1.21
123	1.33	1.54	1.50	1.21	0.99	0.88	1.12	1.12
125	1.29	2.83	2.25	0.92	1.75	0.87	1.25	0.96
Control								
1546	3.92	14.92	23.45	4.08	3.08	2.12	6.71	3.50
1547	4.58	19.66	21.39	3.92	4.62	1.83	5.50	2.63
1548	4.5	17.67	22.46	6.45	2.42	1.88	4.71	2.75

mixture. We concluded that live trypanosomes were incapable of activating bovine complement but autolyzing parasites released potent complement activators. To establish whether this finding was peculiar to *T. congolense* and bovine complement, we constructed dose–response curves for *T. congolense* and *T. lewisi* with human, guinea pig, and bovine complements and found that *T. lewisi* could activate bovine complements 15 times as efficiently as *T. congolense* (20×10^7 *T. congolense* or 1.25×10^7 *T. lewisi* were required to activate 2 CH_{50} units of bovine complement). Similarly 5×10^7 *T. lewisi* could activate 2 CH_{50} units of either human or guinea pig complement, whereas up to 20×10^7 *T. congolense* failed to activate the equivalent amount of guinea pig complement and could only consume 1 CH_{50} unit of human complement. To establish where in the cascade the trypanosome activation of complement took place, C1 was separated from bovine serum. Trypanosome autolysates were then reacted with either C1 or C2 to C9 (the remainder of the serum after C1 had been removed) followed by the addition of the missing component(s). Bovine C1, but not C2 to C9, was activated by the trypanosomes, whereas no activation took place in the controls. This finding has been confirmed (J.O. Minta personal communication), and the interaction has been shown to be at the C1s subcomponent level of human complement.

To ascertain whether or not the observed activation was due to rat-antibody–trypanosome complexes, we treated autolyzed or ultrasonically radiated trypanosomes with 10% final concentration trichloroacetic acid. An aliquot of disrupted trypanosomes was separated by centrifugation into a supernate and a pellet. The abilities of these fractions to activate complement were then tested at various temperatures and hydrogen ion concentrations; heating had no effect on either the supernate

Table 3. Relative average immunoglobulin and complement component levels in serum and nasal washings 38 days after onset of infection.

Immunoglobulin	Source	Ring area (mm^2)		Significance[a] (P value)
		Normal	Infected	
M	Serum	78.5	123.6	0.05
	Nasal washings	16.5	27.3	NS
G1	Serum	264	240	NS
	Nasal washings	41.5	25.0	NS
G2	Serum	248	228.6	NS
	Nasal washings	78.0	63.0	NS
A	Serum	33.0	16.6	0.05
	Nasal washings	83.0	56.6	NS
C1	Serum	115.5	95.3	NS
	Nasal washings	17.5	21.3	NS
C3	Serum	140	94.0	0.05
	Nasal washings	20.0	18.5	NS

[a] NS = not significant.

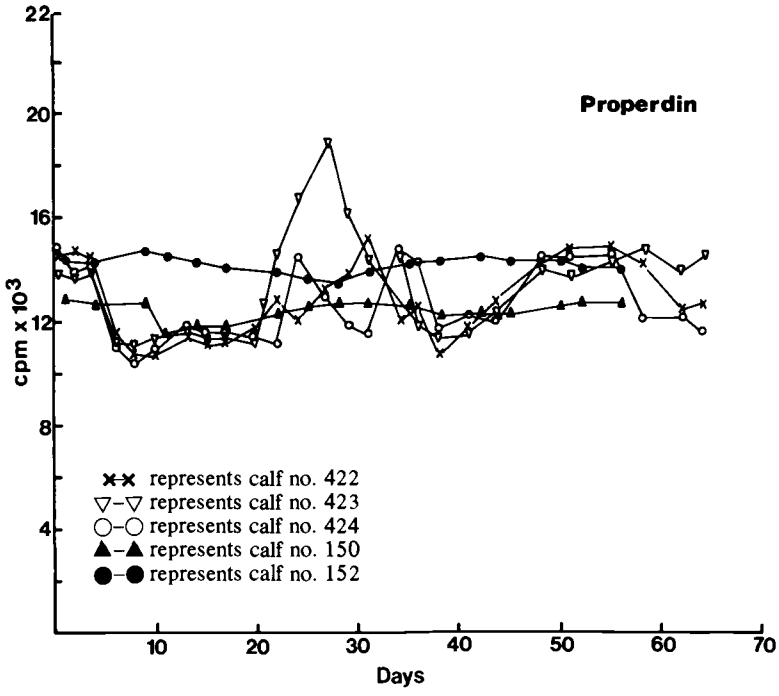

Fig. 5. Time-course plot of serum levels of properdin measured in radioimmunodiffusion assays for calves infected with T. congolense *(422, 423, and 424) and for normals (150 and 152) (from Nielsen, Sheppard, Holmes et al. in press).*

or the trichloroacetic acid preparation, although the trypanosome pellet was partly sensitive to heating at 100 °C. These results indicate that at least two different components are involved and that the heat-labile material is probably a protein, perhaps an enzyme. We further tested the pellet by treating it with a 0.1% (w/v) final concentration of phenylmethylsulfonylfluoride, a broad specificity enzyme inhibitor, which reduced its complement-activating ability by 22%. As the majority of the biological activity resided in the trypanosome supernate and was not affected by the trichloroacetic acid, these steps were used as preliminary purification procedures. Using chromatography, we studied the treated trypanosome material with Sephadex G200 and on DEAE cellulose. Gel

Table 4. *T. lewisi* parasitemias and hemolytic complement levels of rats.

Group	Mean parasites/ml ± SD[a]	Complement levels before *S. typhimurium* infection (Mean ± 1 SD)[a]	Complement levels 48 h after *S. typhimurium* infection (Mean ± 1 SD)
1	$6.01 \times 10^9 \pm 2.54 \times 10^9$ (10)	1.0 ± 1.41 (10)	0.6 ± 0.97 (10)
2	$1.39 \times 10^9 \pm 0.76 \times 10^9$ (10)	7.0 ± 4.35 (10)	ND[b]
3	$1.38 \times 10^9 \pm 0.60 \times 10^9$ (10)	7.2 ± 2.53 (10)	8.0 ± 1.00 (10)
4	–	1.2 ± 2.14 (10)	1.33 ± 1.15 (3)
5	–	1.2 ± 2.14 (10)	0.4 ± 0.84 (10)
6	–	20.0 ± 6.47	22.4 ± 7.93 (9)
7	–	24.0 ± 11.31 (10)	23.6 ± 11.18 (10)
8	–	0 (6)	0 (6)
9	–	0 (6)	ND[b]
10	$5.83 \times 10^9 \pm 3.41 \times 10^9$ (6)	0 (6)	0 (6)

[a] Number within parentheses denotes number of animals in the group.
[b] ND = Not done due to 100% mortality within group.

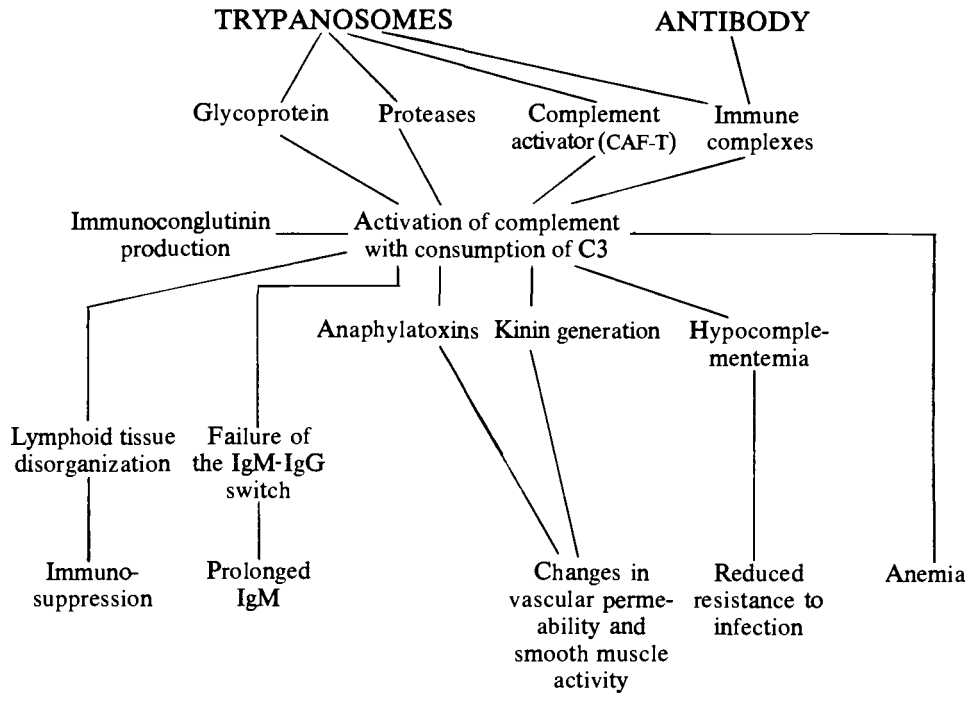

Fig. 6. *Possible mechanism and consequences of hypocomplementemia in trypanosomiasis (from Tizard et al. in press).*

filtration with Sephadex G200 separated in three major populations of molecules as measured in terms of optical density at 214 mm (maximum absorbance). All three fractions contained biological activity thereby not greatly enhancing the purification of the active material. Ion exchange chromatography revealed five populations of molecules measured by optical density at 214 mm but eluted the bulk of the active material in one peak. We attempted to purify the third DEAE cellulose peak further, using thin-layer chromatography and were able to separate nine "spots" of which the biological activity remained near the application point. A spot with an Rf value of 0.057 showed up in electrophoresis on a discontinuous polyacrylamide gel column, resulting in a single anodally migrating band visualized with alcian blue stain.

Attempts to establish the chemical nature of the complement-activating substance have been fruitless; however, hexose determinations of all materials revealed a correlation between the substance's concentration and its ability to activate complement. For instance, a concentration of 4 μg/ml of hexose or greater gave complete activation of 2 CH_{50} units of bovine complement, but lesser concentrations gave a linear relationship between activation of complement and hexose content.

Animals decomplemented with cobra venom factor or the complement activator from trypanosomes and then infected with *T. lewisi* had approximately parasitemias four times as high as did the control groups (Table 4). Decomplemented animals had very low hemolytic serum complement titres compared with those of nontreated controls (group 7). In addition, the *T. lewisi*-infected groups (2 and 3) were partially depleted of complement. Rats that were either decomplemented or infected with *T. lewisi* were also much more susceptible to subsequent infection with *Salmonella typhimurium*. In fact, rats deprived of complement by the trypanosome-activating factor or infected with *T. lewisi* (groups 2 and 10, respectively) began to die 4–5 hours after challenge with freshly cultured *S. typhimurium*, but the controls (group 6) and the cobra venom-treated rats (group 4) did not commence to die until 18 hours after infection. No deaths were observed in the untreated group (6) or in the complement depleted control groups (5 and 8).

Discussion

Some of the possible mechanisms and consequences of hypocomplementemia in trypanosome infection are presented in Fig. 6. Musoke and Barbet (1977) have shown that *T. brucei* possesses

a glycoprotein that is a variant-specific surface antigen capable of activation of human complement by the classical pathway. We (Nielsen and Sheppard 1977; Nielsen, Sheppard, Tizard et al. 1977, 1978a,b) have described complement-activating materials derived from *T. congolense* and *T. lewisi*. We believe these materials contain proteases because they are inactivated by phenylmethylsulfonylfluoride, by trichloroacetic acid, and by heating to 100 °C. We have also demonstrated that both *T. congolense* and *T. lewisi* contain a factor, perhaps a lipopolysaccharide, that activates complement both in vitro and in vivo at the C1 level. Immune complexes may also contribute to consumption of complement in animals infected with *T. brucei* (Lambert and Houba 1974; Murray, P.K. et al. 1974b) or *T. rhodesiense* (Nagle et al. 1974), as complement deposits have been found in kidneys of infected animals. These four possibilities have in common that complement is activated, either by the classical or the alternate pathways or both, and that considerable depletion of C3 occurs.

C3 or complement depletion seriously affects the infected host. The effects include lymphoid tissue disorganization as observed in cobra venom factor depleted animals (Papamichail et al. 1975; Pepys et al. 1976, White et al. 1975). Excess proliferation of plasma cells and disruption of areas of thymus-dependent lymphoid organs have been reported in African trypanosomiasis (Losos and Ikede 1972; Murray, M. et al. 1974; Murray 1974; Terry et al. 1973), possibly interfering with T and B cell cooperation (Terry et al. 1973; Murray, P.K. et al. 1974b). It is, therefore, likely that complement activation in trypanosomiasis is partly responsible for the immunosuppression observed.

Complement depletion in vivo with cobra venom factor has also been shown to cause a decrease in IgG antibody production (Pepys 1972, 1974) and an elevation in IgM antibody production (Nielsen and White 1974). In individuals with Waldenstrom's macroglobulinemia a serum C1q has been reported to correlate directly with IgG levels (Kohler and Muller-Eberhard 1969). In animals experimentally infected with trypanosomes, both C1q and C3 levels are considerably decreased (Nielsen, Sheppard, Holmes et al. in press), and IgM levels are elevated (Nielsen, Sheppard, Holmes et al. in press; Kobayashi and Tizard 1976; Luckins and Mehlitz 1976; Luckins 1972, 1974, 1975; Clarkson et al. 1975). Therefore, complement depletion may interfere with the switch from IgM to IgG antibody, and, along with polyclonal B cell stimulation (Esuruoso 1976; Assoku and Tizard 1978, and Assoku, Tizard, and Nielsen 1977), may lead to an overproduction of IgM. IgG levels are catabolically regulated via the Fc portion of molecule (Fahey and Robinson 1963) and are kept to, at most, a small build-up in complement-deprived animals. Clarkson et al. (1975) and Nielsen, Sheppard, Holmes et al.(in press) found no increase in IgG levels in *T. vivax*- and *T. congolense*-infected animals. This picture, however, is rather inconsistent with the findings of Kobayashi and Tizard (1976), Clarkson and Penhale (1973), and Luckins (1972) who all reported considerably increased IgG levels in trypanosome-infected animals. Pepys (1977) and others (Pepys et al. 1976, 1977) have clearly shown that IgA and IgE classes of antibody were considerably decreased in hypocomplementemic animals. Perhaps, the dramatic decreases of IgA and IgE immunoglobulins in trypanosome-infected calves (Nielsen, Sheppard, Holmes et al. in press) may also be the result of complement activation by the parasite.

Activation of complement is associated with the production of pharmacologic agents, such as anaphylatoxin and kinins, that cause changes in vascular permeability and smooth muscle activity.

Direct complement activation may also be a mechanism in erythrocyte destruction in trypanosomiasis. Thus, erythrocytes may be destroyed as "innocent bystander" cells in the presence of the complement cascade or by the attachment of surface glycoproteins that are capable of activating complement directly (Musoke and Barbet 1977). This suggestion is supported by the findings of Kobayashi and Tizard (1976) who detected C3 on the erythrocytes of *T. congolense*-infected animals.

Finally, the disturbances of the functional lytic complement system and, thereby, deprivation of one of the important nonspecific defence mechanisms leave trypanosome-infected animals highly susceptible to secondary infections with bacteria (Nielsen et al. 1978c). These findings confirm previous observations on increased susceptibility of trypanosome-infected animals to secondary infections (Losos and Ikede 1972) and to tumours (Ackerman and Seed 1976a).

Although the true mechanisms by which the trypanosome can influence the immune system are complex and not well understood at present, hypocomplementemia, as caused directly or indirectly by the parasite, is of considerable consequence in the disease syndrome.

Acknowledgments

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Biologically active lipids generated by autolysis of *T. congolense*

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Abstract. When *T. congolense* is permitted to autolyze at 20 °C for at least 10 hours, it generates active phospholipase A1, which acts on endogenous phosphatidyl choline to generate free fatty acids, the most significant of which are linoleic and palmitic. It is hypothesized that this process occurs in vivo following immune cytolysis. Phospholipase A is capable, in conjunction with free fatty acids, of causing damage to cells and of degranulating mast cells. Although the free fatty acids cause hemolysis in vitro, they are unlikely to do so in vivo because this activity is blocked by serum albumin. Linoleic acid is a potent immunosuppressive agent, and palmitic acid is capable of acting as a B-lymphocyte stimulant in vivo and in vitro. The trypanosome phospholipase and its products are, therefore, capable of inducing a number of lesions characteristic of trypanosomiasis and may represent a major pathogenic mechanism in the disease.

In 1907, Landsteiner and Raubitschek undertook some investigations into *T. equiperdum* and showed that a trypanosome suspension acquired the capacity to lyse sheep erythrocytes following overnight incubation in the cold. They made some preliminary studies on the nature of the hemolysin and concluded that it was probably a lipid.

Since that time, the study of biologically active factors from trypanosomes has proceeded erratically. Inconsistent results and the manifest lack of toxicity of suspensions of living trypanosomes eventually led to the conclusion that trypanosome toxins did not exist (Goodwin 1974). However, in 1975 Huan reported that he could generate hemolytic activity in suspensions of *T. brucei*, *T. gambiense*, *T. vivax*, and *T. congolense*. The hemolytic material appeared to be associated with a protein of molecular weight about 10000 daltons. Unfortunately, the published data do not make clear either the viability of the trypanosomes or the time required for hemolysin generation.

Trypanosome Autolytic Products

In 1976, while studying the phlogistic properties of *T. congolense* suspensions, we found that although suspensions of freshly isolated organisms caused inflammation in rabbits commencing about 10 hours following intradermal inoculation, suspensions of organisms autolyzed for 10 hours before injection produced an immediate inflammatory response (Tizard and Holmes 1976). When the suspensions were mixed with washed sheep erythrocytes, the fresh organisms took about 10 hours to cause hemolysis, whereas autolyzed organisms produced hemolysis within seconds. The suspensions were equally toxic for nucleated cells, such as rabbit buffy coat cells or mouse peritoneal macrophages.

We found that hemolytic activity was generated after approximately 9 hours incubation of a suspension containing 3×10^9 *T. congolense*/ml (Fig. 1). The active material showed some anomalous behaviour. Its generation did not require cations, but the hemolytic activity was calcium dependent. Heating at 100 °C for 15 minutes effectively prevented its generation but appeared to have no effect on the existing hemolysin. The possibility of proteases being involved in this activity was minimized by the finding that neither trasylol nor

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Source of Free Fatty Acids

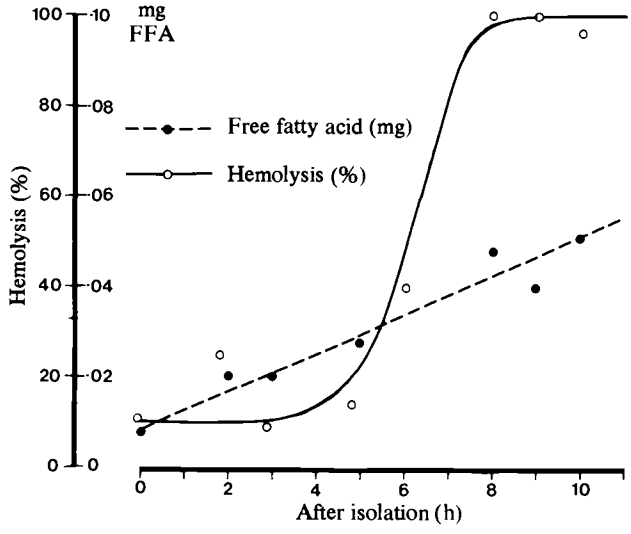

Fig. 1. Generation of free fatty acid and the appearance of hemolysin in an autolyzing suspension of T. congolense *(from Tizard et al. 1978).*

soybean–trypsin-inhibitor influenced its activity or its generation.

Subsequent experiments indicated that hemolytic activity could be extracted from the chloroform suspension by chloroform-methanol (Tizard et al. 1977). When we used chromatography to study a chloroform-methanol extract of these organisms on silica gel and then analyzed the fractions, we found most of the hemolytic activity was in the free fatty acid-containing fraction although some was attributed to lysophosphatidyl choline and phosphatidyl ethanolamine (Fig. 2). Our finding that the hemolytic material was free fatty acid was supported in tests with bovine serum albumin, which blocked hemolysis and cast doubt on the potential significance of the hemolytic activity as a cause of anemia in vivo.

Gas-liquid chromatography of the purified free fatty acids (FFA) indicated that they were a complex mixture containing at least 15 components. The predominant acids were palmitic, stearic, and linoleic, which each accounted for approximately 25% of the total (Table 1). There was no detectable arachidonic acid, but the mixtures contained a significant quantity of γ-linolenic acid. Hemolytic and cytotoxic assays of each of the major fatty acids in the mixture indicated that linoleic acid accounted for most of the hemolytic activity. Studies on both the trypanosome autolysate and on artificial fatty-acid mixtures supported this observation (Tizard et al. 1978).

Sequential chromatography of chloroform extracts of autolyzing *T. congolense* demonstrated that the levels of phosphatidyl choline (PC) in the suspension gradually declined while the FFA gradually increased (Tizard et al. 1978). The suspension became hemolytic when FFA levels exceeded 0.2 mg/ml (Fig. 1). From this observation, we hypothesized that the FFA were derived from PC through the activities of a phospholipase A (E.C. 3.1.1.4) rather than by the hydrolysis of triglycerides by lipases. Phospholipase A activity was measured by estimating the hydrolysis of

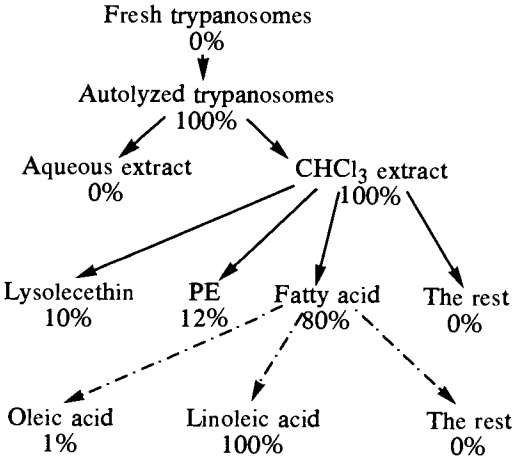

Fig. 2. Identification of T. congolense *hemolysin by sequential chloroform extraction, thin layer chromatography, and titration of free fatty acids. Percentages are the degree of hemolysis of a suspension of 2.5% washed sheep erythrocytes; the free fatty acids were tested at concentrations identical to those found in trypanosome autolysate.*

^{32}P-labeled PC to lysophosphatidyl choline (LPC) and water-soluble products. Lipase activity was estimated by measuring the hydrolysis of glycerol-tri(1-^{14}C)palmitate. We found that both fresh and autolyzed trypanosome suspensions were devoid of lipase activity and that freshly isolated *T. congolense* contained little phospholipase activity (3.1 nmole PC hydrolyzed/mg protein/h), although the latter rose on autolysis to reach as high as 300 nmole/mg/h (Tizard et al. 1978). By using other labeled substrates, such as 2(9,10-di^{3}H)dipalmitoyl PC and ^{32}P-1 acylglycerolphosphatidyl choline, we showed that the phospholipase A activity was of the A1 type. In addition, we found evidence of lysophospholipase A1 (E.C. 3.1.1.5) with an activity of 0.5 nmoles/mg/h (Roberts et al. 1977). Lysophospholipase activity did not increase significantly on autolysis (Fig. 3).

The existence of the two enzymes suggests that the hydrolysis of PC to water-soluble products takes place in three stages (Fig. 4). First, phospholipase A1 converts PC to LPC by removing the fatty acid at the 1-acyl position; second, the fatty acid in the second position probably spontaneously switches into the first position; finally, the lysophospholipase removes the second fatty acid, leaving glycerylphosphoryl choline. (We also have tentative evidence for the existence of a phosphodiesterase, which may remove the choline, leaving glycerylphosphate.)

Table 1. Fatty acid content of *T. congolense* hemolysin as determined by gas-liquid chromatography.

Chain length	Name	%
14:0	Myristic	1.0
16:0	Palmitic	20.0
16:1	–	1.4
17:0	Margaric	1.8
18:0	Stearic	24.6
18:1	Oleic	7.5
18.2	Linoleic	22.5
18:3ω6	γLinolenic	2.8
18:3ω3	Linolenic	0.2
20:0	Arachidic	3.1
20:7	–	2.8
21:2	–	2.4
22:0	Behenic	2.1
22:4	–	1.7
22:6ω3	–	2.7

Consequently, trypanosome death and autolysis generates relatively high levels of phospholipases

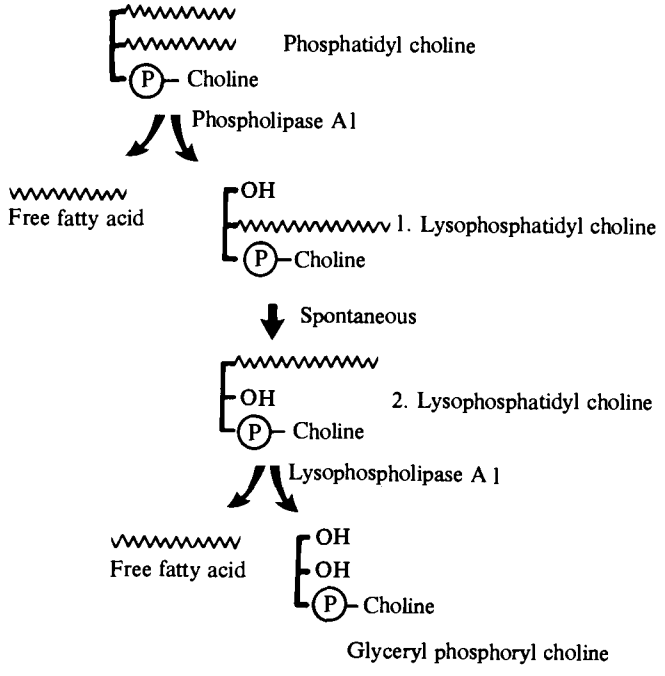

Fig. 3. *Sequential hydrolysis of trypanosome phosphatidyl choline on autolysis.*

and free fatty acids, which have significant biological activity and probably contribute to the pathogenesis of the trypanosomiases.

Phospholipases: Phospholipases are capable of acting on cell-membrane phospholipids to cause cell destruction or hemolysis (Fig. 4) (Colley et al. 1973), but purified phospholipase A1 is, by itself, only weakly hemolytic. It is apparently unable to penetrate the membrane and to reach the membrane phospholipids; however, in combination with surface active molecules such as free fatty acids that act upon cell membranes, it can cause hemolysis (Condrea, De Vries, and Mager 1964). *T. congolense*, which accumulates in the microcirculation, may cause endothelial cell damage as has recently been reported for *Corynebacterium ovis* (Carne and Onon 1978). *T.b. brucei* may autolyze within connective tissue, damage nearby fibroblasts, and lead to vascular permeability resulting in edema (Goodwin 1974). Injection of trypanosome autolysates containing phospholipases increases vascular permeability and provokes a local inflammatory response (Musoke and Barbet 1977; Seed 1969; Tizard and Holmes 1977). This may be associated with local vascular damage, but it is likely to occur as a result of phospholipase-induced mast cell degranulation.

Autolyzed but not fresh suspensions of *T. congolense* are capable of degranulating mouse mast cells both in vitro and in vivo. In the absence of purified trypanosome phospholipases, it is not possible to prove that they are responsible, but it is known that phospholipases derived from other sources are well capable of provoking mast cell degranulation (Orr and Cox 1969).

The lysophospholipase of *T. congolense* is also noteworthy because it is potentially capable of destroying LPC and may be responsible for the dramatic drop in serum LPC levels observed in *T. congolense*-infected cattle. LPC is required for the utilization of cholesterol and other lipids, and its absence, by blocking lipid utilization, may cause an animal to mobilize its lipid reserves and, thus, cause severe loss of condition, so characteristic of the trypanosomiases (Roberts et al. 1977).

Free Fatty Acids: Soon after we identified free fatty acids as the hemolysins of autolyzed trypanosomes, we realized that serum albumin effectively blocks the hemolytic process in animals with a low parasite burden, for example, cattle infected with *T. congolense* (Tizard, Holmes, and Nielsen 1978a) in which FFAs do not occur free in serum. In support of this is the lack of evidence of intravascular hemolysis in *T. congolense*-infected cattle (Fiennes 1970; Goodwin 1974).

Free fatty acids, when administered in relatively large doses to experimental animals can induce thrombosis (Connor, Hoak, and Warner 1963), myocardial lesions (Willebrands, Ter Welle, and Tasseron 1973), thrombocytopenia (Shore and Alpers 1963), and hypoglycemia (Fig. 5), the last of which is due to stimulation of insulin secretion. All these lesions occur in trypanosomiasis (Losos and Ikede 1972), but there is no evidence available to show that they are directly due to trypanosome FFAs.

Although free fatty acids bound to serum albumin are not cytolytic, they remain capable of influencing lymphocytes in such a way that an infected animal's capacity to mount an immune response may be modified. Of the FFAs present in trypanosomes, the one that has been most extensively studied from the immunologic point of view is linoleic. This acid constitutes 20–25% of the total FFA in both *T. congolense* and *T. brucei* (Tizard et al. 1978) and it is a potent immunosuppressive agent. In vitro, it can inhibit the lymphocytes' responses to PHA, PPD tuberculin, and to antigen (Mertin 1976; Mertin and Hughes 1975;

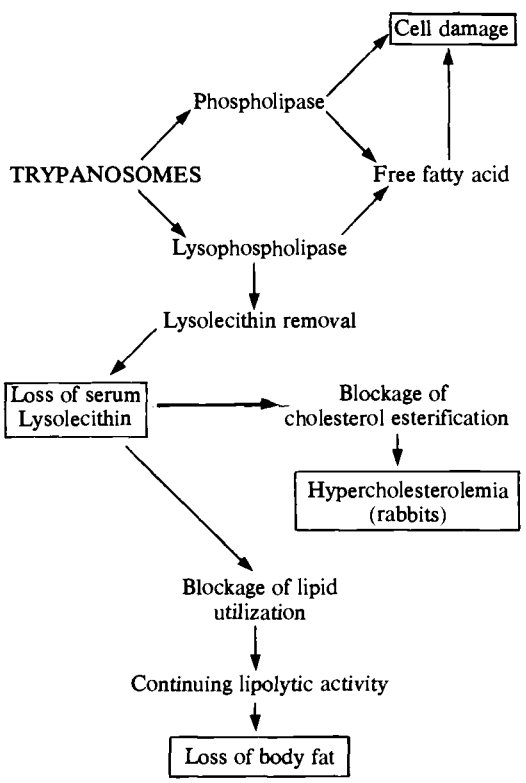

Fig. 4. *The potential biological activities of trypanosome phospholipases.*

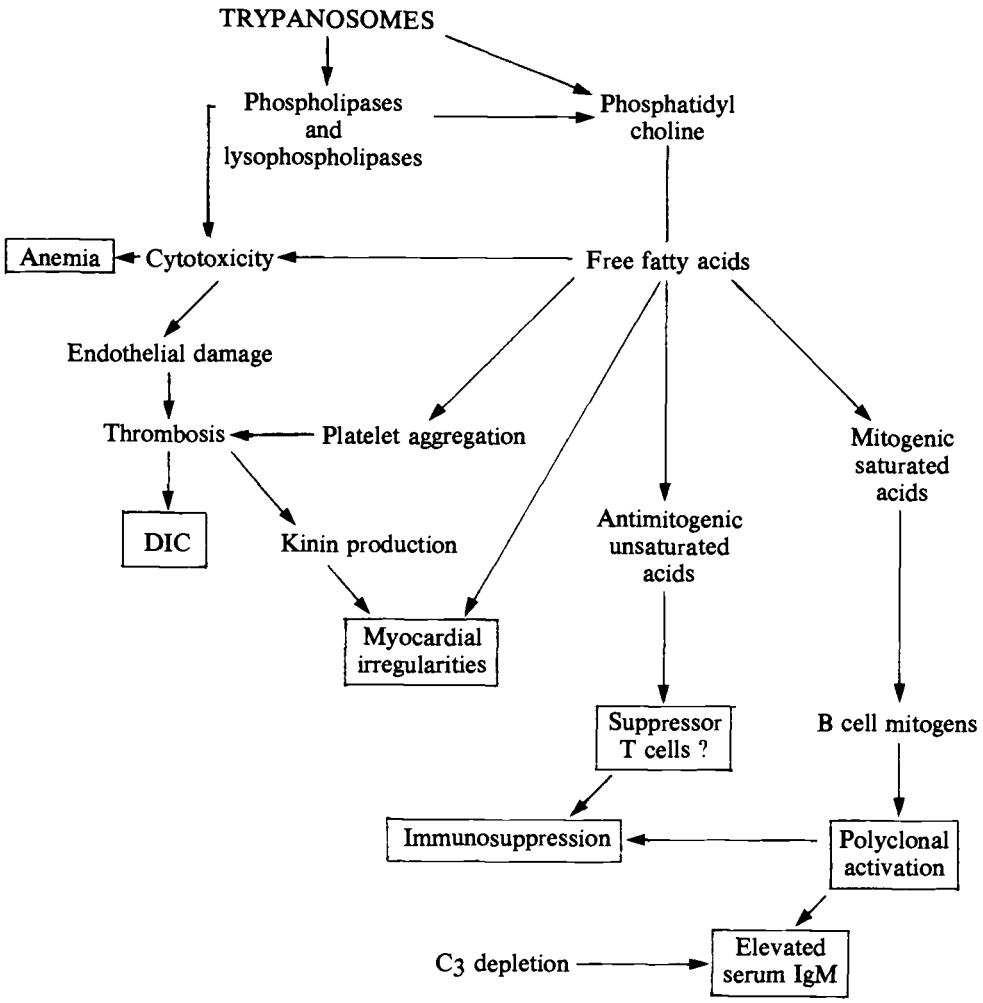

Fig. 5. *The potential biological activities of trypanosome-derived free fatty acids (from Tizard et al. 1978).*

Mertin et al. 1974) and is used to prolong the survival of skin allografts (Mertin 1976; Mertin and Hughes 1975). Autolyzed *T. congolense* suspensions in subhemolytic doses can also inhibit PHA incorporation by lymphocytes (Assoku and Tizard 1978). In addition, a mixture containing FFAs in proportions identical to those found in *T. congolense* and at a concentration equal to that in 4×10^9 organisms (a relatively large dose) significantly suppresses the immune response of mice to sheep erythrocytes (Fig. 6).

Free fatty acids also disrupt the architecture of the spleen and lymph nodes, decreasing the proportion of white pulp and depressing its uptake of tritiated uridine (Meade and Mertin 1976) in a manner identical to that observed in trypanosomiasis (Losos and Ikede 1972).

Although linoleic acid is immunosuppressive, autolyzed *T. congolense* in a subsuppressive dose stimulates thymidine uptake by mouse lymphocytes. The target cells of this activity are probably B cells, as the cells of nude mice respond fully and those from cyclophosphamide-treated mice do not respond (Assoku and Tizard 1978).

Using fractionation techniques identical to those described for the hemolysins, we have shown that the mitogenic activity is found in the FFA fraction of a chromatographed chloroform-methanol extract of autolyzed *T. congolense* (Fig. 7). When the four major FFAs in this mixture were tested at concentrations corresponding to those in the autolysate, we found that both palmitic and stearic acids were mitogenic. Palmitic acid was the more potent of the two (Fig. 8) (Assoku, Hazlett, and Tizard sub-

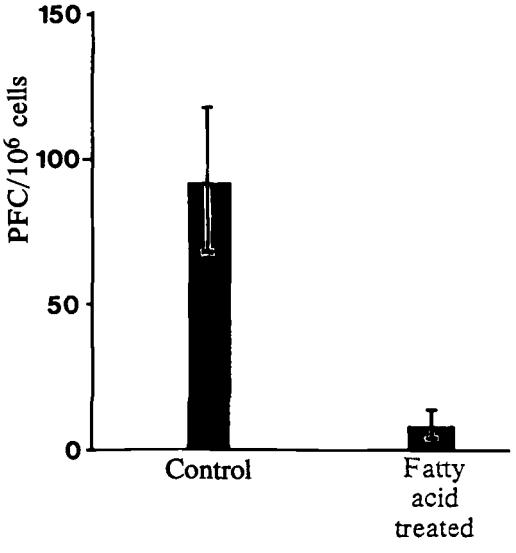

Fig. 6. Immunosuppressive effect of a free fatty acid mixture similar to that generated by autolyzing trypanosomes.

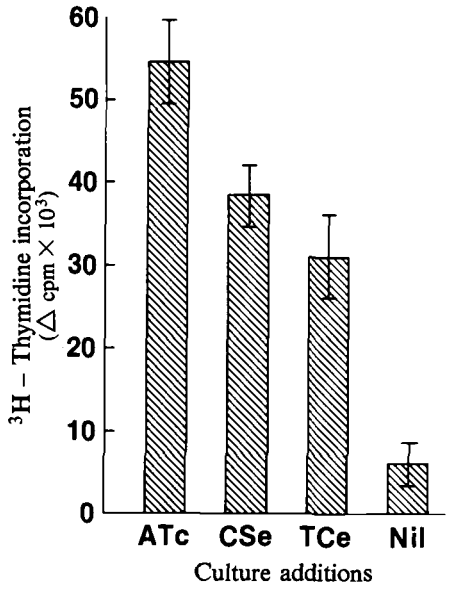

Fig. 7. Mitogenic activity of T. congolense *autolysates (ATc), chloroform soluble extracts (CSe), and the free fatty acid component separated on thin layer chromatography (TCe) on mouse spleen cells (from Assoku, Hazlett, and Tizard submitted for publication).*

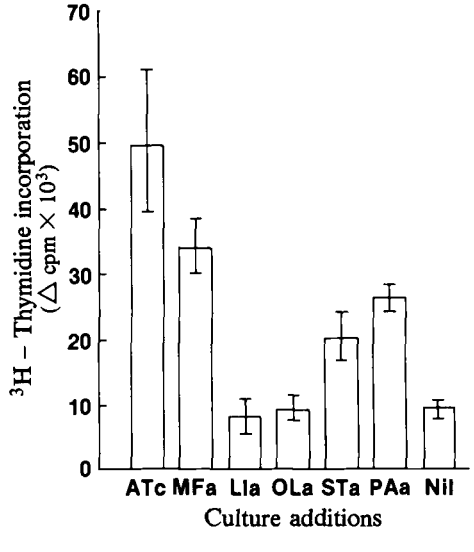

Fig. 8. Mitogenic activity of autolyzed T. congolense *(ATc), a fatty acid mixture of similar composition (MFa), and individual free fatty acids (Lla, linoleic; OLa, oleic; STa, stearic; and PAa, palmitic) at concentrations identical to those in the trypanosome autolysate (from Assoku, Hazlett, and Tizard submitted for publication).*

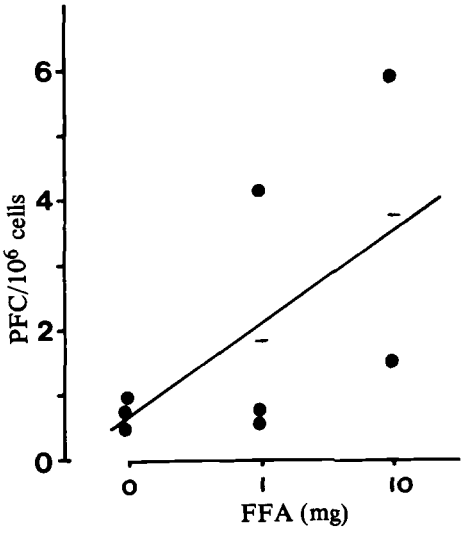

Fig. 9. Background PFC in the spleens of mice following a single intravenous dose of palmitic acid.

Table 2. Production of hemolysins and mitogens by different trypanosomes.

Trypanosome species	Activable phospholipase	Free fatty acid release	Cytolytic autolysate	Mitogenic autolysate
T. congolense (TREU 112)	+ + +	+ + +	+ +	
T. brucei (Shinyanga III)	+ + +	+ + +	+ + +	+ +
T. musculi	+	+	−	+
T. lewisi	+	+	−	+
T. theileri	−	(+)	(+)	−

mitted for publication) and in subsequent tests appeared to be responsible for immunostimulatory activity of trypanosome autolysates (Fig. 9). This finding is supported by our recent observation that a single dose of palmitic acid provokes an increase in background IgM PFC within 30 minutes. An increase in background IgM PFC is also a characteristic of mice infected with *T. congolense* (Hazlett and Tizard 1978).

In summary, free fatty acids may exert opposing effects on B lymphocytes — linoleic and possibly other unsaturated acids being immunosuppressive and palmitic and, to a lesser extent, stearic being immunostimulatory.

Other Trypanosome Species

The mere occurrence of biologically active factors in autolyzing trypanosomes does not make them important. In the absence of quantitative in vivo data, it is necessary to assess their importance independently. One method is to relate them to pathogenicity by examining a number of trypanosome species (Table 2).

In addition to *T. congolense*, we have examined *T. brucei* and the "nonpathogenic" trypanosomes *T. musculi, T. lewisi*, and *T. theileri*. *T. brucei* possesses an activable phospholipase, generates free fatty acids on hemolysis, and is known to possess B-cell mitogenic activity (Fig. 10). *T. musculi*, although its phospholipase is only slightly activated and it generates small amounts of FFA (insufficient for hemolysis), is mitogenic for mouse B cells but not as powerfully as is *T. congolense*. *T. lewisi* has properties similar to *T. musculi* (Tizard et al. 1978a). *T. theileri* does not possess an activable phospholipase and does not generate hemolytic FFA. So far, we have been unable to demonstrate any mitogenic activity of *T. theileri* on bovine peripheral blood lymphocytes. In general, the possession of an activable phospholipase appears to be associated with pathogenicity.

Discussion

It is well recognized that aging of mitochondria in mammalian cells results in loss of respiratory control and subsequent phospholipase activation. It is tempting to postulate a similar phenomenon in

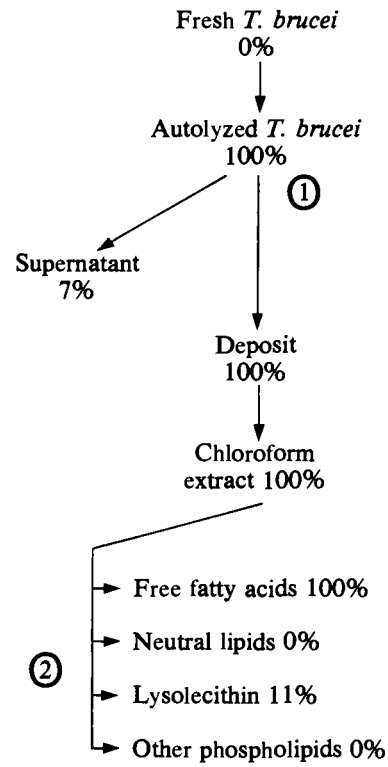

Fig. 10. *Identification of free fatty acids as the hemolysins of* T. brucei *(from Tizard, Holmes, and Nielsen 1978a).*

the salivarian trypanosomes. For instance, the bloodstream forms of *T. brucei* possess a relatively large tubular mitochondrion that could provide a source of phospholipase (Vickerman 1974). The role of this enzyme in the trypanosome is not clear, but it may be related to the process of mitochondrial proliferation and regression (Newton, Cross, and Baker 1973). Although the stercorarian trypanosomes also possess a mitochondrion, it appears to be less well developed, and we know of no evidence that it undergoes the same dramatic changes as in the salivarians.

In considering other differences that distinguish the pathogenic from the nonpathogenic trypanosomes, we believe that the pathogenic organisms proliferate to high levels and are repeatedly destroyed in large numbers. Also, both clinically and pathologically, the disease appears to be exacerbated during proliferation. Thus, infected animals appear to sicken, may suffer a hemolytic crisis (Friedberger and Szymanowski 1911), and show fever peaks during immune trypanolysis (Seed and Vamey 1976; Woo and Kobayashi 1975). Similarly, a shock syndrome of the Herxheimer type is associated with drug-induced trypanolysis (Ormerod 1970).

We conclude that the pathogenicity of African trypanosomes is associated not primarily with the presence of living organisms in the bloodstream but with the prolonged exposure of animals to the products of dead and disrupted organisms. We, therefore, hypothesize that at least some of the lesions induced by both *T. brucei* and *T. congolense* arise from trypanosome death and subsequent autolysis and from exposure to trypanosome-derived phospholipases and free fatty acids.

Pharmacologically active substances in *T. vivax* infections

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Abstract. We researched two pharmacologically active substances — bradykinin and serotonin — which are known to occur in animals with trypanosomiasis. Attempts were made to correlate the action of bradykinin and serotonin in vitro, with their actions in vivo in noninfected animals. Then, we experimentally infected heifers and goats with *T. vivax* and monitored the animals' physiologic changes (heart frequency, rectal temperature, ruminal motility) as well as their levels of bradykinin and serotonin, but we did not detect any substantial correlation between them.

In our study, my colleagues and I at the Institute of Tropical and Protozoan Diseases and at the Institute of Veterinary Pharmacology and Toxicology in the Netherlands experimentally infected animals with a mouse-infective *T. vivax* strain Y58, described by Leeflang, Buys, and Blotkamp (1976), and attempted to link the animals' changes in blood serotonin levels and bradykinin activity with the observed clinical and pathological changes. We also compared dose-dependent inhibition of rumen motility and possible change of heart rate (caused by intravenous administration of bradykinin and serotonin) with the blood levels of serotonin and bradykinin activity and the observed clinical and pathological changes in our infected animals.

Bradykinin

Earlier studies have shown that bradykinin (BK) and its natural derivatives kallidin (Lys-BK) and Met-Lys-BK cause:

- Contraction of the nonvascular smooth muscles (bronchi, stomach, intestines, uterus),
- Increased capillary permeability (Lewis 1970),
- Lowered blood pressure due to arteriolar, capillary, and venular dilation, and tachycardia, which is probably caused by a reflex mechanism (Trautschold 1970),
- Relaxation of the rat's duodenum in vitro (Horton 1959; Gaddum and Horton 1959),
- Pain (when injected intradermally) (Armstrong 1970),
- Migration of leukocytes (Lewis 1962), and
- Tachyphylaxis (following repeated administrations) (Armstrong 1970; Collier 1970).

We undertook studies of bradykinin on ruminal strip smooth muscle tone and on ruminal contractions. In vitro (0.2 ng/ml) it caused a ruminal strip preparation to contract, and in vivo (0.5μg/kg intravenously injected into goats) it caused an inhibition of the reticuloruminal contractions (Van Miert 1970; Veenendaal unpublished data). These results may reflect an enhanced reticuloruminal smooth muscle tone causing reflex inhibition of the normal cyclical movements. Reflex inhibition occurs when the high-threshold tension receptors in the reticuloruminal wall, innervated by vagal sensory fibres, are tonically (continuously) active (Leek 1969).

How *T. vivax* activates bradykinin release is not clear; however, elevated levels of the substance are found very early in infection. In the early stages at least, therefore, its activity is probably not due to immune complexes, as suggested by Boreham and Wright (1976a). It may be, however, that immune complexes are responsible for elevated levels that can be observed later. Boreham and Wright (1976a) did not exclude the possibility that the trypano-

somes trigger the bradykinin activity; this view gains some support from work in which Seed (1969) and Tizard and Holmes (1977) were able to derive vasoactive materials from trypanosomes.

In cattle we infected with *T. vivax*, we found the highest level of bradykinin (60 ng/ml blood) at 4 days post infection. Levels gradually declined after that until about day 20, when an increase was noted and a rise–fall pattern began to emerge (Van den Ingh, Zwart, Van Miert et al. 1976; Van den Ingh, Zwart, Schotman et al. 1976).

When we attempted to correlate these findings with pathological and clinical changes that have been attributed to bradykinin activity and/or trypanosomiasis, our results were inconclusive.

Our studies of BK had suggested that uninfected animals experience inhibition of ruminal motility during increased bradykinin activity, but our studies in the *T. vivax*-infected animals did not bear this out. We observed no correlation between a rise in bradykinin levels, fever, and decreased ruminal motility (Veenendaal et al. 1976), and the literature on trypanosomiases contains no suggestion of greatly decreased appetite. Shien et al. (1975) observed that dogs infected with *T. evansi* maintained a good appetite even during periods of high parasitemia and fever. Similar observations were made by Mamo and Holmes (1975) in cattle infected with *T. congolense*. In general, gastric secretion and hunger contractions (reticuloruminal contractions in ruminants) are absent in animals with fever and anorexia is present as long as the fever persists (Van Miert in press).

Similarly, we were unable to correlate raised bradykinin levels with increased capillary permeability, although the condition has been noted in studies of both bradykinin and trypanosomiasis. At any rate, there are many factors that may contribute to vascular leakage in trypanosomiasis, only one of which is bradykinin.

For instance, vasoactive amines, such as histamine and serotonin, are likely chemical mediators of vascular leakage. In addition, fibrinopeptides, which are liberated from fibrinogen molecules and certain fragments of the complement system, i.e., C3a and C5a, are known to induce vascular leakage (Ryan 1974; Eisen and Vogt 1970). In trypanosomal infections, fibrinopeptides may be produced by disseminated intravascular coagulation or by complement, activated by trypanosomes. The latter possibility gains some support from studies of Musoke and Barbet (1977) who have described activation of the complement system by a variant antigen of trypanosomes. Another possibility is that the platelets or fibrinothrombi formed during trypanosomal infections directly affect the vessel wall and that they cause anoxia of the cells and tissues, resulting in increased vascular permeability.

Free fatty acids and reduction of lysophosphatidyl choline have also been mentioned as a cause of vasopermeability, but their role in vivo is not clear (Tizard and Holmes 1976; Roberts and Clarkson 1977). Another possibility is the low albumin content or heart failure in trypanosomiasis.

We would, therefore, hesitate to consider the observed vascular permeability in our animals and laboratory studies as only due to bradykinin, although a local effect of the substance cannot be excluded.

The story was much the same for blood pressure changes and tachycardia. In *T. vivax*-infected goats, there was no correlation between bradykinin levels and pulse rate, although this finding does not rule out hypotension. For example, in *T. brucei*-infected rabbits, Wright and Boreham (1977) found hypotension without increased heart rate.

The poor, or nonexistent, correlations between the clinical signs of *T. vivax* and the pattern of bradykinin levels are puzzling and cannot be explained in terms of tachyphylaxis. In an attempt to do so, we injected goats intravenously four times with bradykinin (2 μg/kg body weight) at 30-minute intervals before and after infection with *T. vivax*; there was no marked difference in response.

Serotonin

Some of the effects that have been observed after intravenous injection of bradykinin can also be found after vast intravenous injection of serotonin. Serotonin is another pharmacologically active substance formed during trypanosomiasis. It causes lowered blood pressure accompanied by tachycardia, dilatation of the vascular bed, and contraction of the main vascular smooth muscles (intestines, stomach, and bronchi). Tachyphylaxis, too, is common after repeated serotonin infusions (Szabuniewicz and McCrady 1977). Our work in goats showed serotonin decreased rumen motility but did not affect heart rate.

Little is known about the mechanism that activates serotonin during trypanosomiasis. It is known that serotonin is produced by several cells but not the thrombocytes, which only store the substance. When aggregation occurs, thrombocytes are destroyed, and they release the serotonin, which is either broken down very rapidly or taken up again by various other cells. Thus the serotonin in whole blood is a measure of thrombocyte destruc-

tion: when the thrombocytes are destroyed, blood serotonin levels drop.

During trypanosomal infections, the thrombocytes may be lowered by intravascular platelet aggregation or by disseminated intravascular coagulation. Studies to date suggest that both mechanisms operate but that one of them assumes a dominant role. For example, we found that thrombi consisted mainly of fibrin (Van Dijk, Zwart, and Leeflang 1973) in acute *T. simiae* infections and in the late stages of *T. vivax* infection in mice, whereas mainly platelet aggregation was found in goats dying during the acute stage of the infection (Van den Ingh et al. 1976a,b). Platelet aggregation also occurs in surviving animals, although the pathologic effects of it seem to depend largely on whether the reticuloendothelial system (RES) is able to remove the aggregations from the circulation (Van den Ingh et al. 1976a,b).

The fall in blood serotonin during temperature peaks, which are associated with peaks of parasitemia, and the presence of many platelet-thrombi in goats dying during overwhelming parasitemia suggested to us a correlation between *T. vivax*, parasitemia, platelet aggregation, blood serotonin decrease, and fever (Veenendaal et al. 1976, Van den Ingh et al. 1976a,b). Our studies suggested that *T. vivax*, either dead or alive, did not directly affect goat thrombocytes but immune complexes of *T. vivax* and antibody induced serotonin release (Slots et al. 1977). The serotonin released from platelets is rapidly destroyed or taken up by certain cells in vivo, and whole blood serotonin is a valuable parameter of thrombocyte destruction. We believe a systematic effect is unlikely, however. When we infused two normal goats with 0.5 μg serotonin/kg bodyweight for 300 minutes, which approximates the serotonin released during a thrombocytic crisis in trypanosomiasis, there was a slight increase in ruminal motility and in the breakdown product of serotonin, 5-hydroxy indoleactic acid (5-HIAA) in the urine; the latter, however, was not found in *T. vivax*-infected goats. This may be an indication that the uptake of released serotonin plays a role during *T. vivax* infections.

These experiments do not exclude the possibility of a local effect of serotonin, e.g., in the lungs, during trypanosomiasis.

Conclusions

It should be realized that release of mediators, complement activation, platelet aggregation, blood clotting, and tissue damage are all interrelated (Eisen and Vogt 1970; Ryan 1974). Most investigators have studied only a single component of these interrelated reactions during trypanosomiasis. Very little is known about how the different mediators potentiate or mitigate each other or about the role of the trypanosomes themselves. Moreover, the in vitro activities of these mediators may differ markedly from their effects on living animals, and their local effects may not be systemic.

How little we know about this ability to modify the chain of reactions is demonstrated by our studies of flurbiprofen, a potent anti-inflammatory and antipyretic agent, which inhibits platelet aggregation induced in vitro by epinephrine, collagen, and thrombin. This drug inhibited the febrile reactions in *T. vivax*-infected goats but did not prevent or reverse the associated drop in blood serotonin level. Moreover, it was apparent that flurbiprofen had a deleterious effect: all the goats died with a disseminated intravascular coagulation found at postmortem examination (Van Miert et al. 1978).

Pharmacologically active substances in *T. brucei* infections

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Abstract. Pharmacologically active substances appear to be important in the pathogenesis of African trypanosomiasis. Urinary kallikrein increases in rabbits infected with *T. brucei* 3–4 days after infection but the mechanism is unknown. Plasma kallikrein is also activated with peak levels occurring 10–14 days after infection. Levels of circulating IgG immune complexes parallel the release of kallikrein, and it has been demonstrated in vivo and in vitro that immune complexes activate the kallikrein-kinin system probably by the absorption and activation of Hageman factor. Activation of Hageman factor causes not only the release of kinin mediators of inflammation but also activation of the coagulation, fibrinolytic, and complement systems that contribute to the pathogenesis of the disease. Increases in blood viscosity contribute to the hemodynamic changes seen in trypanosomiasis.

Human trypanosomiasis and the experimental disease caused by *T. brucei* are basically inflammatory conditions. Rabbits infected with *T. brucei* suffer inflammation after about 2 weeks, with their extremities such as ears, eyelids, external nares, and scrotum, being particularly affected. It is not surprising, therefore, to find that the pathogenic mechanisms are very similar to the inflammatory response and that the mediators of inflammation are involved in this disease.

During chemotherapeutic studies, Goodwin noted that nontoxic doses of drugs cured the parasitemia of some mice infected with trypanosomes but that often the animals died within 24 h or so (Goodwin and Richards 1960). This phenomenon reminded him of an earlier study by Stephan and Esquibel (1929) who had similar results when using acriflavine to treat cattle with piroplasmosis. Goodwin set out to investigate this phenomenon in *T. brucei* infections, trying to explain the pathogenic mechanisms of trypanosomiasis to suggest better methods of treatment.

Release of Kinins and Kallikrein

The earliest studies demonstrated increases in histamine and short-chain peptides related to bradykinin in the urine, blood, and tissues of infected mice (Goodwin and Richards 1960; Richards 1965). Similar results were also obtained in patients with severe burn trauma (Goodwin et al. 1963), suggesting that tissue damage was important. The release of pharmacologically active substances was not confined to trypanosomiasis, and similar results were obtained with mice infected with other protozoal infections, bacteria, and viruses (Goodwin and Richards 1960). At about the same time as these studies were undertaken, the kallikrein-kinin system was being extensively studied, and many new facets about the biochemical pathways and mechanisms of activation were being discovered. More importantly, the kallikrein-kinin system was being implicated in various disease processes, especially anaphylactic shock (Brocklehurst and Lahiri 1962), inflammation (Elliott, Horton, and Lewis 1960), rheumatic disease (Eisen 1970), and bacterial infections (Amundsen and Rugstad 1965).

Kinins were found to be released in rabbits and cattle infected with *T. brucei* and humans infected with *T. rhodesiense* (Boreham 1968a, 1970). Levels of the $\alpha 2$ globulin precursor kininogen decreased at the same time as kinin was detected in the blood. Similar studies in malaria and babesiosis showed that the kallikrein-kinin system was involved in these diseases as well (Onabanjo and Maegraith 1970; Wright 1973). In chronic trypanosomiasis infections, the release of kinins occurred 1–2 days after the first antigenic variant

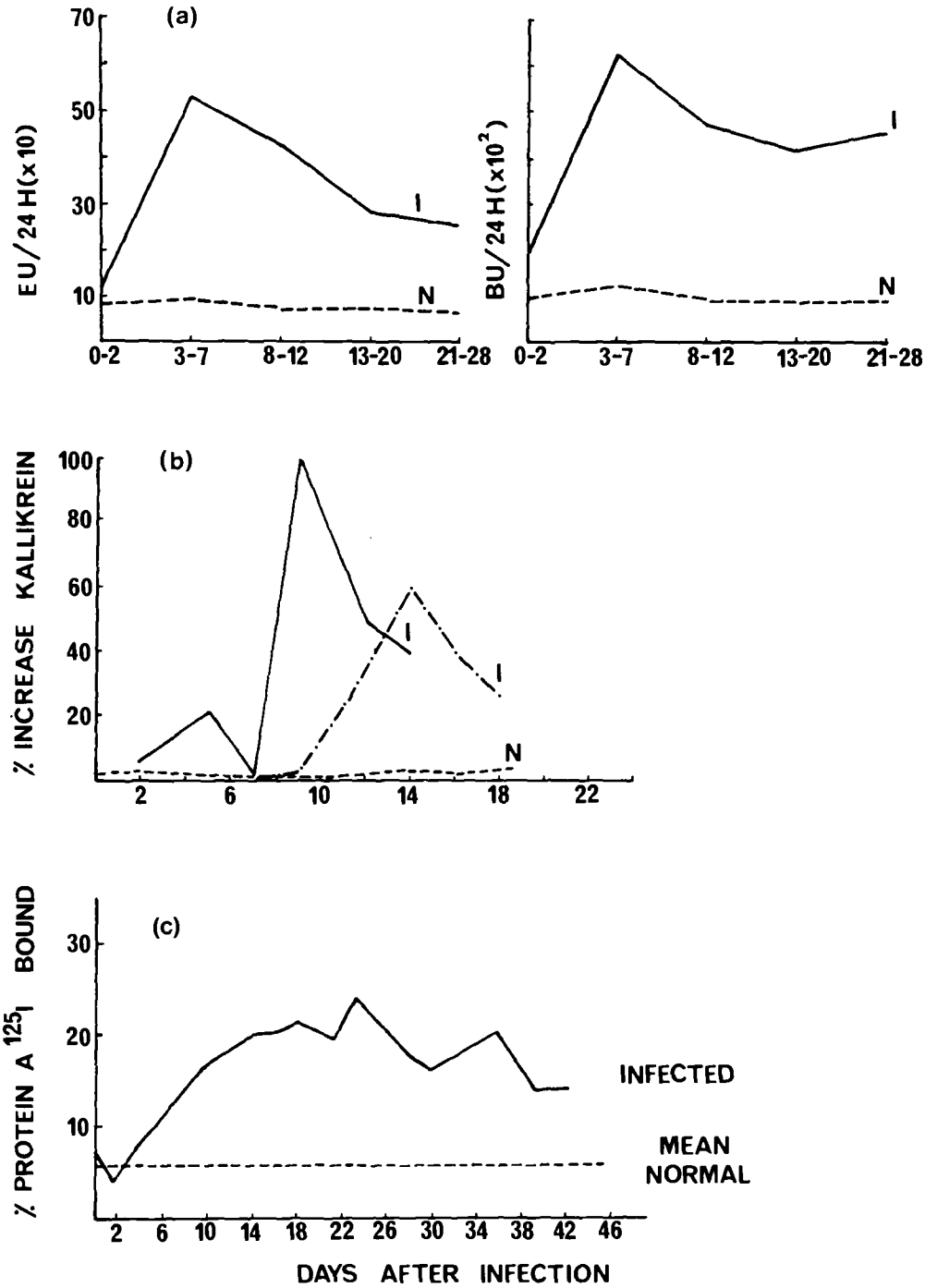

Fig. 1. Changes in urinary kallikrein (a), plasma kallikrein (b), and circulating IgG immune complexes (c) in rabbits infected with T. brucei.

of trypanosomes was produced, suggesting that there was an association between the two events (Boreham 1968a).

Kallikreins are enzymes normally present in inactive forms and found in the glandular organs, blood, lymph, and urine. Although similar in properties, the enzymes are different from one another. They have direct biological activities, such as chemotaxis, and they act on kininogen to form other biologically active substances — kinins. Plasma kallikrein cleaves high molecular-weight kininogen to form bradykinin, whereas glandular kallikrein forms kallidin (lysyl-bradykinin).

Samples of urine from rabbits infected with *T. brucei* have been analyzed for kallikrein activity at intervals during the infection (Fig. 1a); the results indicate that within 3–4 days of infection, urinary kallikrein activity increases and peaks at 4–8 times normal values after 6–10 days. Despite subsequent fluctuations, concentrations remain high throughout the infection (Wright and Boreham 1977).

Plasma kallikrein concentrations have also been measured, but they show a different pattern of results (Fig. 1b). Increases in plasma kallikrein occur after rises in urinary kallikrein and reach a peak 10–16 days after infection. Although the length of time varies, maximum plasma kallikrein concentrations are always detected after the urinary kallikreins are raised. This indicates that increases in urinary kallikrein do not occur as the result of increased filtration of plasma kallikrein through the kidney glomerulus. This is confirmed by studying the properties of urinary and plasma kallikreins released in trypanosome infections. Plasma kallikrein but not urinary kallikrein is inhibited by soybean–trypsin-inhibitor, whereas both plasma and urinary kallikreins are inhibited by the protease inhibitor aprotinin (Trasylol). Plasma prekallikrein levels show an inverse relationship to plasma kallikrein.

Mechanism of Kallikrein Activation

The evidence that immune complexes activate plasma kallikrein has recently been reviewed (Boreham and Wright 1976b). The most important pieces of evidence are that complexes cause the release of kinins in vitro from a kininogen substrate and that injection of immune complexes in vivo into rabbits causes a profound hypotension, which is inhibited by aprotinin. Trypanosomal antigen injected intravenously into rabbits with preformed antibody due to an infection also results in hypotension caused by the activation of the kallikrein-kinin system (Boreham and Wright 1976a,b). It seems probable that immune complexes absorb and activate Hageman factor (factor XII), which in turn activates prekallikrein. This is supported by in vitro evidence that heating the kininogen substrate to 65 °C but not 56 °C prevents kinin release by immune complexes. (Hageman factor is destroyed at 65 °C but not 56 °C.)

The author measured the concentration of circulating immune complexes in infected rabbits; he used staphylococcal protein A, which binds to the Fc portion of the immunoglobulin G molecule, as the basis of a radioimmunoassay for soluble IgG complexes (Crawford and Lane 1977). He found that the amount of circulating immune complexes increases significantly during infection (Fig. 1c), and the increase corresponds to the activation of kallikrein. Preliminary studies using SDS gel electrophoresis suggest that the antigen component of the immune complexes is nontrypanosomal in origin. This finding is, perhaps, not surprising because only about 5% of the IgG antibody produced in trypanosomiasis is trypanosome-specific (Freeman et al. 1970) and several auto- and heterophile antibodies are also present (Boreham and Mackenzie 1974). It is now well established that in *Babesia* infections of cattle, an esterase enzyme present in the parasite is responsible for kallikrein activation (Wright 1975). This enzyme, which will also convert fibrinogen to fibrin, probably acts directly on prekallikrein and not through an intermediate substance.

Although kallikrein is also activated during malaria infections, no attempts have been made to determine the mechanism. Because malaria is an intracellular parasite, one might expect a mechanism similar to *Babesia* infections. In vivo and in vitro experiments have demonstrated that a similar esterase enzyme does not occur in *T. brucei*.

Because urinary kallikrein concentrations are raised within about 3 days of infection, it seems unlikely that immune complexes are involved in its activation. Urinary kallikrein is known to be produced in the kidney, probably in the juxtaglomerular complex (Nustad 1970). Various mechanisms may be hypothesized to explain its release, such as circulating endogenous chemicals, increased kidney blood flow (possibly caused by plasma kinins released locally), or alterations in aldosterone concentrations (Keiser et al. 1976).

Kinins normally have a very short half-life (less than one complete circulation of the blood) due to the presence of kininase enzymes (Ferreira and Vane 1967), but they exist much longer when kininases are inhibited by slightly acid pH (Edery and Lewis 1962). In trypanosomiasis, the inflam-

matory exudates, which are acidic, may be responsible for local concentrations of free kinins.

No work on other pharmacologically active substances in *T. brucei* infections has been published since the review of Boreham and Wright (1976a). Because trypanosomiasis produces a great deal of stress to the host, one would expect catecholamine to be released (Goodwin 1976); however no depletion of epinephrine from the adrenal glands of rats was found during *T. brucei* infections. Only Yates (1971) has reported some depletion of norepinephrine from the hearts of rabbits chronically infected. The catecholamine metabolites do increase, but the significance of these changes is not known at present. It appears that changes in the catecholamines are certainly not as important in *T. brucei* as they are in *T. cruzi* infections. It has recently been shown that *T. cruzi*-infected rats experienced complete depletion of norepinephrine in the heart during the acute phase of infection and recovered during the chronic phase (Machado, Machado, and Gomes 1975; Machado, Machado, and Chairi 1978). This finding implies complete functional denervation of the adrenergic nervous system in acute Chagas' disease.

The importance of histamine and 5-hydroxytryptamine (5HT) as pharmacologic mediators varies with host species and tissue. The rabbit is very insensitive to histamine, whereas the guinea pig responds well to both agonists. There is no evidence to suggest that these substances are important in human or cattle disease caused by *T. brucei* subgroup organisms. At present, the kallikrein and kinins seem to be the most important pharmacologically active substances released. Almeida et al. (1977) showed that the hypotensive undecapeptide substance P is released in *T. cruzi* infections, but so far no similar studies have been undertaken in *T. brucei* infections. The roles of prostaglandins, angiotensin, and SRS-A have not yet been evaluated, although SRS-A has been implicated in acute systemic anaphylaxis in cattle (Eyre, Lewis, and Wells 1973) and may be an important mediator of pathogenic reactions in cattle trypanosomiasis.

Possible Effects of Kallikrein Activation

Kallikrein is activated early in *T. brucei* infection in rabbits, cattle, and humans and likely initiates a number of pathogenic processes. The kallikrein-kinin system is interrelated with the coagulation, fibrinolytic, and complement systems (Fig. 2). Activation of Hageman factor initiates reactions in all four systems. Thus, it is possible that the activation of Hageman factor by immune complexes accounts for the majority of the pathological symptoms. Many of the biochemical pathways activated are likely to produce secondary pathological changes in tissues and organs.

The biological effects of the kallikrein-kinin system are hypotension; increased capillary permeability; pain; chemotaxis of leukocytes; contraction of many isolated smooth-muscle preparations; and the release of catecholamines, histamine, and prostaglandins.

Severe hypotension is characteristic of rabbits infected with *T. brucei* and occurs early in infection (Boreham and Wright 1976a). Hypotension is not a common finding in human trypanosomiasis, although reports do exist (Sicé 1937; Buyst 1975). The most likely explanation of this anomaly is that the maintenance of blood pressure is a complex mechanism consisting of hormonal and neural components in which a balance is normally maintained. Thus, compensatory mechanisms exist to counteract the effects of kallikrein.

Increased capillary permeability is a consistent feature of trypanosomiasis. Kinins, like histamine and 5HT, bear a net positive charge on their molecules and probably combine with acidic groups on mucopolysaccharides and glycoprotein components of blood vessel walls, initiating changes in permeability. The consequences of altered permeability are great. Not only are plasma and lymph constituents allowed to escape from vessels but the quality of the fluid is also changed. For example, it has been shown that in inflammatory conditions the amount of protein in lymph increases from 1 to 8%. Early in the inflammatory reaction, tissue degeneration occurs, causing the release of metabolic substances that are mainly acidic. These substances raise the osmotic pressure outside blood vessels so much that the fluid within is withdrawn to dilute them, and edema results. Degeneration of tissues in trypanosomiasis has been demonstrated by Goodwin (1970, 1971).

One of the consequences of inflammation is the production of the acute phase beta globulin proteins known as C-reactive protein (CRP). Concentrations of CRP indicate the severity of the inflammation and the amount of damage caused. In trypanosomiasis, CRP levels have been shown to be raised in rabbits infected with either *T. congolense* or *T. brucei* (Thomasson et al. 1973; Cook 1979). The anti-inflammatory drug indomethacin prevents the increase in CRP levels and delays the onset of pathological symptoms caused by edema.

Plasma albumin decreases in trypanosomiasis. This may be the result of increased plasma volume (Boreham 1967; Clarkson 1968; Naylor 1971), or it

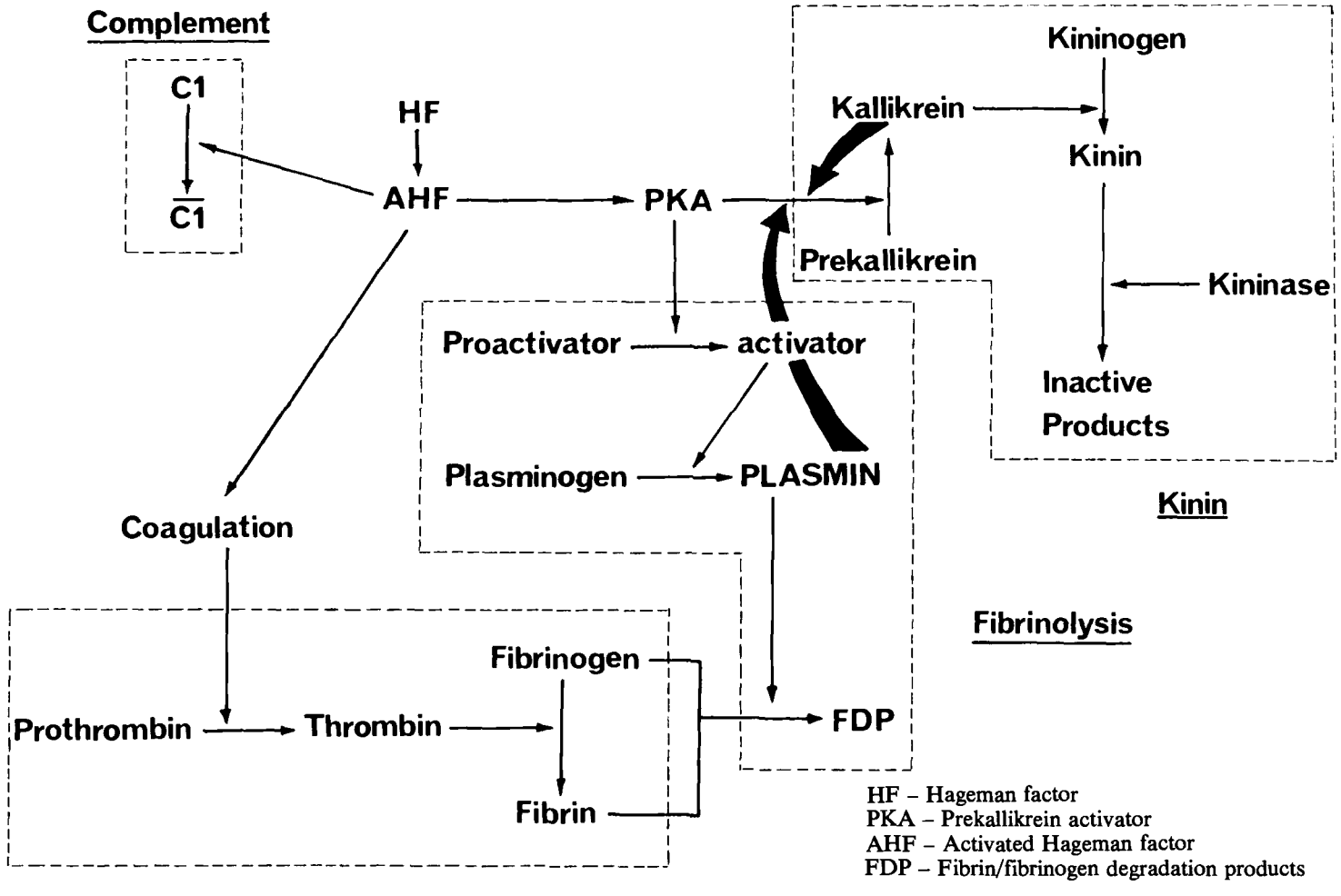

Fig. 2. Interrelationship of the kallikrein-kinin system with the coagulation, fibrinolytic, and complement systems. The solid arrows indicate feedback mechanisms.

may be due to depressed hepatic synthesis of albumin in inflammation (Page 1972). Because albumin is the major factor controlling the colloid osmotic pressure within blood vessels, its decrease would encourage edema.

Changes in coagulation have been noted during trypanosomiasis, although the effects are probably not very significant. Thrombocytopenia occurs (Davis et al. 1974; Robins-Browne, Schneider, and Metz 1975), and increases in some of the clotting factors, especially factors VIII and XII, have been reported (Boulton, Jenkins, and Lloyd 1974). Robins-Browne and Schneider (1977) reported that four out of five patients suffered from bleeding disorders early in trypanosomiasis infections and, interestingly, that suramin sodium initially aggravated the coagulation defects. The latter finding may be attributable to the drug's inhibiting kallikrein and complement activation and preventing the action of thrombin on fibrinogen (Eisen and Loveday 1973).

The fibrinolytic system is also activated in trypanosomiasis; at the same time, plasma fibrinogen increases significantly, contributing to the hyperviscosity syndrome seen in infected rabbits (Facer 1976). Fibrinolysis occurs in rabbits, demonstrated by the formation of breakdown products (FDP) of fibrin and fibrinogen as a result of plasminogen activation (Boreham and Facer 1974). The presence of FDP has also been reported in human trypanosomiasis (Greenwood and Whittle 1976a).

Several authors have shown decreases in C3 levels, indicating activation of complement in trypanosomiasis (Nagle et al. 1974; Greenwood and Whittle 1976b). Such activation results in the formation of chemotactic fragments C3a, C5a, and the C5b67 complex as well as the anaphylatoxic mast cell degranulating fragments C3a and C5a. One of the most important components produced when complement is activated is C3b, which enhances phagocytosis.

Chemotaxis of leukocytes is an important process in inflammation. It is known that the conversion of prekallikrein to kallikrein results in the formation of chemo-attractants for both neutrophils and human blood monocytes (Gallin and Kaplan 1974). Recent studies (Cook 1977) have indicated that in vitro immune complexes of trypanosomes and antibody, but not trypanosomes alone, generate chemotactic activity that is not complement-dependent. Trypanosomes and immune sera sensitize isolated tissues to agonists such as bradykinin, acetylcholine, and histamine (Boreham and Wright 1976b). One possible explanation of this observation is that trypanosomes or their products cause a local release of prostaglandins.

The major event in the pathogenesis of trypanosomiasis caused by *T. brucei* subgroup organisms is the activation of Hageman factor by immune complexes. This occurs approximately 10 days after infection when sufficient antigen and antibody are present. Activated Hageman factor initiates chain reactions that produce the signs and symptoms of trypanosomiasis, the most important of which seems to be the alteration in capillary permeability. The hyperviscosity syndrome that occurs as a result of changes in red-cell structure and increased plasma globulins and fibrinogen causes sludging of the blood in the microvessels and further kinin activation with the formation of microthrombi. When immune complexes are produced, they have direct pathological effects as they are deposited in the glomerulus (Houba 1977). The pathogenesis of trypanosomiasis is not unique but is similar in many respects to other inflammatory diseases such as rheumatoid arthritis and glomerulonephritis.

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Discussion summary

P.F.L. Boreham and F.E.G. Cox

The manifestations of cellular injury in trypanosomiasis are many and varied and arise in a number of different ways. Variations occur in different hosts and with different trypanosome species. Although there may be common underlying mechanisms of pathogenesis, different abnormalities may result because of the predominance of one or more of the mechanisms. The main lesions are anemia with *T. congolense* and *T. vivax*, and generalized inflammation with the *T. brucei* subgroup organisms.

For many years, it has been suggested that "toxins" may be involved in the pathology of trypanosomiasis, and now, in vitro at least, there are three possible candidates: a hemolytic factor that may contribute to the anemia; phospholipases that may cause vascular endothelial damage and immunodepression; and complement-activating factors that may be responsible for some cell damage. Anemia, hypocomplementemia, and immunodepression are common to all pathogenic trypanosome infections of humans and cattle. A hypothesis compatible with available information on the complement-activating factor is that it could be a glycoprotein that forms an integral part of the membrane of the trypanosome but is normally masked by the surface coat and is freed only when the trypanosome dies. The sources of the hemolytic factor and the phospholipase are also in the parasite, but the actual location is unknown.

Immune complexes appear to play a central role in the inflammatory reaction by causing the release of pharmacologically active substances especially kallikrein and, from platelets, 5-hydroxy-tryptamine. These complexes also activate complement and may play a part in renal manifestations that occur as secondary events in some forms of trypanosomiasis.

In rabbits infected with *T. brucei*, increases in whole blood, plasma, and serum viscosities occur. These correlate with changes in red cell stickiness, increased fibrinogen, and macroglobulins. In calves infected with *T. congolense*, there is also increased blood viscosity, sludging of the blood, and widespread microvascular dilation. These important observations indicate that detailed hemodynamic measurements of cardiac output, intracapillary pressures, structural changes, etc., are needed to evaluate the effect on the vascular system of a range of infections.

So many different interacting and sequential events occur in trypanosomiasis that the actual outcome of any particular infection will depend on which of the pathological pathways breaks first. Furthermore, under field conditions, animals may be infected with more than one species of trypanosome, and the resulting pathologic consequences may take a variety of forms. The possibility of using drugs such as aprotinin to ameliorate the more generalized symptoms of trypanosomiasis deserves attention.

Anemia of bovine African trypanosomiasis: an overview

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Abstract. The cardinal sign of African trypanosomiasis in the bovine is anemia. Based on the presence or absence of the parasite and on clinical and pathological findings, we have divided the disease into three phases. During phase one, the parasitemic phase, anemia is largely hemolytic, resulting from increased red blood cell destruction by phagocytosis; splenomegaly is a feature. Factors possibly involved in this process include hemolysis produced by the trypanosome, immunologic mechanisms, fever, disseminated intravascular coagulation, and an expanded and active mononuclear phagocytic system. Throughout phase two and three, in the apparent absence of trypanosomes, erythrocyte destruction continues, possibly due to the expanded mononuclear phagocytic system. During phase two, there is evidence of dyshemopoiesis and by phase three it is marked, possibly reflecting a defect in iron metabolism related to trapping in the mononuclear phagocytic system. The last phase is characterized by massive hemosiderosis, a yellow gelatinous inactive marrow, and a small spleen. The infection during any of the three phases may cause congestive heart failure and death due to a combination of anemia, circulatory disorder, and myocardial damage. Some animals survive with a persistent low grade anemia and are stunted or wasted, whereas others, particularly trypanotolerant breeds, may make a complete clinical recovery following the apparent elimination of the parasite.

The cardinal sign and major disease-promoting factor of bovine African trypanosomiasis is anemia (Hornby 1921; Murray, M. 1974). Despite its importance, it has many unexplained aspects about which reports frequently appear to conflict. Too often, in discussing the kinetics and mechanisms of the anemia, authors consider only the static situation rather than the progression of the disease. My overview of the development of anemia is meant to reconcile the different views. It includes cumulative experience of ILRAD studies on cattle infected by needle challenge with bloodstream forms of trypanosomes and on cattle exposed to natural tsetse fly challenge (Fig. 1). In Fig. 1, no specific values have been included, only estimates, as the major parameters of anemia, parasitemia, and time vary with breed, challenge, previous exposure, nutritional status, age, parturition, etc. The disease may also be affected by the virulence and species of trypanosome involved, although in our experience mixed infections of *T. congolense*, *T. vivax*, and *T. brucei* are more common than single infections in natural field conditions. Based on the presence or absence of trypanosomes and on clinical and pathological findings, we have divided the disease into three distinct, but overlapping, phases.

Phase One

The first phase may last from 3 to 12 weeks, during which death may occur. The onset and severity of the anemia is directly related to the appearance of the parasite in the blood and to the level of parasitemia (Murray, P.K., Murray, Wallace, et al. in press a, in press b). There is now considerable evidence that the anemia during this phase, is hemolytic (Mamo and Holmes 1975; Holmes 1976; Preston and Wellde 1978; Dargie et al. in press) and that red-cell destruction is mainly phagocytosis by the expanded and active mononuclear phagocytic system (MPS) (Murray, M. 1974; Murray, M., McIntyre, Murray et al. in press b). Splenomegaly is marked, partially as a result of massive red-cell sequestration and erythrophagocytosis as well as striking lymphoid hyperplasia. Erythrophagocytosis is not confined to the spleen and is widespread throughout the liver, lungs, hemal nodes, bone marrow, and circulation.

Evidence that hemodilution is involved in the development of anemia (Fiennes 1954; Naylor 1971; Holmes 1976; Valli, Forsberg, and McSherry 1978) has now been refuted by Dargie et al. (in

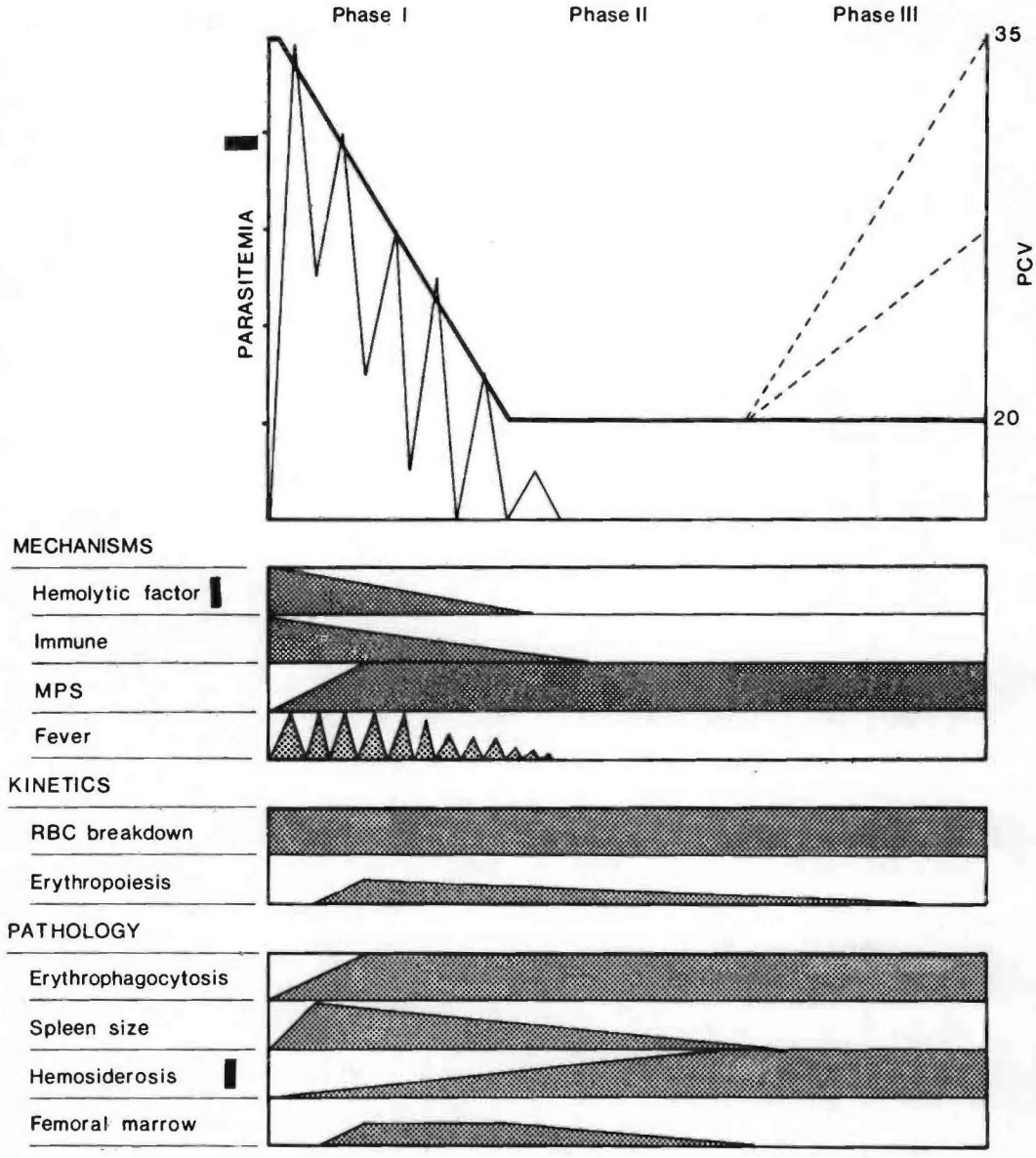

Fig. 1. Anemia of bovine African trypanosomiasis in relation to parasitemia, time, underlying mechanisms, erythrokinetics, and postmortem findings. Hatched areas represent an estimation of the importance of a reaction at any one time. Total time approximates 1 year and is based on a single needle challenge with bloodstream forms of trypanosomes or a tsetse fly challenge limited to a few months of the year such as occurs in the Gambia.

press) whose findings have important clinical implications and establish that measurement of packed red cell volume (PCV) provides a reliable index of the degree of anemia. That the bone marrow is responsive has been confirmed by ferrokinetic studies (Mamo and Holmes 1975). Furthermore, although the anemia is usually normocytic normochromic, we have observed, in individuals, a macrocytic response, a change also seen by Valli, Forsberg, and McSherry (1978), and, frequently, we have found an increase in red marrow in longitudinal sections of the femur. Occasional petechial and ecchymotic hemorrhages may be found scattered throughout the carcass, but

widespread hemorrhage is not a feature, apart from a distinct syndrome involving *T. vivax* in which massive hemorrhage predominates.

Phase Two

If cattle survive phase one, they pass into a second phase that lasts for several months and is characterized by transient and scanty parasitemias (often parasites cannot be detected) and by a persistent low grade anemia.

Erythrocyte destruction by the expanded and active MPS continues, although splenomegaly is no longer a consistent feature (Dargie et al. in press; Murray, M., McIntyre, Murray et al. in press b). Ferrokinetic studies have confirmed that there is increased erythropoiesis but not to the extent expected in relation to the degree of anemia (Dargie et al. in press). The femoral marrow response varies from animal to animal and frequently shows little obvious change. A significant new feature is increased hemosiderin deposition throughout the body. Animals with a PCV of 20% or less may die during this phase.

Phase Three

Provided cattle do not die or become reinfected during phase two, they pass into another stage of the syndrome that may be ongoing or may end in the animals' death or recovery. This phase is characterized by the absence (admittedly difficult to confirm) of parasites in blood and tissues. Because of the implication of this finding, namely that the disease continues in the absence of trypanosomes, we intensively examined cattle in this phase for the presence of parasites. One study involved 47 cattle (37 Ndama and 10 Zebu) that had previously been infected with *T. congolense* and *T. brucei* and had then been subjected to challenge by *Glossina palpalis*. We took both jugular and peripheral blood samples and checked them for parasites using the highly sensitive blood buffy coat darkground phase contrast technique (Murray, Murray, and McIntyre 1977); we also subinoculated cattle blood into laboratory rodents. Necropsy was conducted on 10 cattle and tissues were inoculated into laboratory rodents. For 90 days, and in many cases longer before the termination of this study, no trypanosomes were detected in any animals. A significant proportion of cattle made a slow but complete clinical recovery as judged by hematological values and clinical condition; more Ndama than Zebu behaved in this way. Other cattle made a partial recovery with PCVs remaining between 25 and 30%. However, despite the absence of detectable parasites for several months, one group made no clinical improvement and had persistently low PCV levels (20–25%).

These experimental studies have been confirmed by longitudinal epidemiological investigations of some 2000 cattle over 5 years, which indicate that this chronic trypanosomiasis syndrome, i.e., a persistent low grade anemia in the absence of trypanosomes, is common. Animals may die, but many remain alive in poor health characterized by wasting or stunting.

Although the erythrokinetics and ferrokinetics of the chronic trypanosomiasis syndrome remain to be investigated, our postmortem findings give some indication of what is going on, namely, continued red cell destruction, iron trapping, and dyshemopoiesis. Thus, although splenomegaly is not a feature, erythrophagocytosis is still found throughout the body. Hemosiderosis is widespread in the spleen, lungs, liver, and bone marrow, and the femoral marrow is often yellow, gelatinous, and inactive.

We believe that the underlying mechanisms of the anemia can be divided into two categories. In the first are mechanisms that operate when the parasite is present, and in the second are those involved in the apparent absence of the trypanosome. In the earlier parasitemic phases of the disease, trypanocidal drug treatment causes a dramatic return to normal hematological values (Holmes and Jennings 1979; Murray unpublished data), whereas in the last phase of the disease, i.e., phase three, there is usually a poor response to chemotherapy (Murray unpublished data).

During phase one of the disease, when the anemia is due largely to increased red cell destruction by the expanded and active MPS, several mechanisms are possible:

- *Hemolytic factors:* Because a significant drop in PCV occurs within a few days of infection, coinciding with the first parasitemia, and subsequent progress of the anemia parallels waves of parasitemia, several workers have speculated that the trypanosome produces a factor capable of damaging red blood cells. Huan et al. (1975) showed that *T. brucei* produces a protein (molecular weight 10 000 daltons) that is capable of lysing red blood cells, and other investigators demonstrated that *T. congolense* during autolysis generates phospholipase A activity and free fatty acids that can destroy red blood cells (Tizard and Holmes 1976; Tizard, Holmes, and Nielsen 1978a; Tizard et al. 1978). So far, we have found that the major African trypanosomes including *T. congolense*, *T.*

vivax, *T. brucei*, *T. rhodesiense*, and *T. gambiense* are all capable of producing a hemolytic factor or factors (Murray, M., Huan, Lambert et al. in press). We have detected hemolytic activity in fresh freeze-thawed trypanosomes and in material prepared from dying trypanosomes. The hemolytic factor described by Murray et al. (Murray, M., Huan, Lambert et al. in press) is a heat-stable, trypsin-sensitive substance with a molecular weight just less than 12 000 daltons and isoelectric points in different preparations ranging from pH 5.0 to 5.5. In other words, the trypanosomes seem to produce more than one factor capable of causing red blood cell damage — a possibility that requires further investigation.

The most relevant question is whether or not the factors operate in vivo or just in vitro. Our evidence, at least from studies in *T. brucei*-infected rats, suggests that they are operative in vivo, but that the activity is generated mainly by dying trypanosomes. The onset and course of anemia and the development of parasitemia correlate strikingly with the generation of hemolytic activity in the plasma (Fig. 2). Our figures came from plasma samples prepared immediately after collection; these were heated at 56 °C for 1 hour before use. Blood samples that were left on the bench for a few hours or were collected as serum had significantly greater hemolytic activity, a finding that suggests trypanosomes generate hemolytic activity as they die.

• *Immunologic mechanisms:* Even before the turn of the century, there was evidence that immunologic mechanisms were involved in the anemia of African trypanosomiasis; in 1898 Kanthack, Durham, and Blandford reported autoagglutination and increased sedimentation rates in cattle and humans with trypanosomiasis (reviewed by Gall, Hutchinson, and Yates 1957). More recently, Woodruff et al. (1973) have demonstrated immunoglobulin and complement on erythrocytes of a small number of patients using indirect hemagglutination. Zoutendyk and Gear (1951) and Barrett-Connor, Ugoretz, and Braude (1973) had similar results using direct hemagglutination, and Woo and Kobayashi (1975) found some evidence that immunologic mechanisms operate in the development of anemia in rabbits infected with *T. brucei*. Erythrocytes from infected rabbits lyse in the presence of fresh complement, suggesting the presence of antigen–antibody complexes, and antibodies directed against the trypanosomes can be eluted from erythrocytes of infected rabbits. Also, trypanosome antigen is readily absorbed on to normal rabbit erythrocytes, which then lyse in the presence of complement and homologous antisera (Woo and Kobayashi 1975).

Immunoglobulins and C3 have been demonstrated on erythrocytes of calves that have been experimentally infected with *T. congolense* (Kobayashi, Tizard, and Woo 1976), and IgM and IgG with antibody activity against *T. congolense* have been eluted from the erythrocytes. The presence of complement-dependent trypanosome antigen–antibody complexes on erythrocytes of infected animals likely facilitates phagocytosis by the expanded and active MPS via Fc or complement receptors on the macrophage. If elevated immunoconglutinin levels, which are known to occur in African trypanosomiasis (Ingram and Soltys 1960; Woodruff 1973) reflect fixation of complement to erythrocytes, immunoconglutinin may facilitate increased erythrocyte clearance from the circulation by causing agglutination.

• *Fever:* The occurrence of fever, often coinciding with a trypanolytic crisis, is a well recognized feature of African trypanosomiasis, and in vitro studies have demonstrated that red blood cells exposed to temperatures of a few degrees above normal body temperature for a few hours have increased osmotic fragility, undergo accelerated hemolysis, and have a shortened life span in vivo (Karle 1969). It is possible, therefore, that fever plays a role in red cell damage and destruction.

• *Disseminated intravascular coagulation (DIC):* Disseminated intravascular coagulation can lead to hemolytic anemia, termed microangiopathic hemolytic anemia, in which the red blood cells are damaged by widespread fibrin deposition in the microvasculature; the erythrocytes then appear as distorted cells or schisocytes, which are liable to lysis or phagocytosis (Wintrobe 1975). DIC has been described in humans infected with *T. rhodesiense* (Barrett-Connor et. al. 1973; Robins-Browne, Schneider, and Metz 1975), and it may occur in *T. brucei*-infected rabbits (Boreham and Facer 1974). It has also been suggested as a mechanism of anemia in African trypanosomiasis by Jenkins et al. (1974) who describe marked alterations in red cell morphology, including red cell fragments, in *T. brucei*-infected rabbits. Valli, Forsberg, and McSherry (1978) observed some fragmented red cells in cattle infected with *T. congolense*; however, we have found little histopathological evidence that DIC is important in cattle except, perhaps, in the hemorrhagic syndrome caused by *T. vivax*.

• *Role of the mononuclear phagocytic system:*

One of the most striking features of bovine African trypanosomiasis is the expanded and active MPS that develops soon after infection and continues throughout the disease (reviewed by Murray, M. 1974; Murray, M., McIntyre, Murray et al. in press b). It is likely that the condition of the MPS results from the massive intravascular presence of living, dying, and dead trypanosomes as well as antigen–antibody complexes, as the size and activity of the MPS is a direct function of its particulate work load (Jandl et al. 1965). That splenomegaly and an expanded MPS are capable of causing anemia has been shown by studies in which methyl cellulose (Palmer et al. 1953; Zuckerman, Abzug, and Burg 1969), zymosan (Gorstein and Benacerraf 1960), and *Corynebacterium parvum* (Nussenzweig 1967) produced hemolytic anemia. Investigators have suggested that splenomegaly leads to red cell stasis within the spleen, prolonged contact with expanded and active MPS, and hence increased erythrophagocytosis. Morphological and kinetic studies indicate that this mechanism is also operative in bovine trypanosomiasis with an increased rate of removal of normal as well as "damaged" erythrocytes.

It appears that the hemolytic anemia of phase one of bovine African trypanosomiasis depends on the presence of the trypanosome and has a multifactorial etiology that may include hemolytic factors produced by the trypanosome, immunologic mechanisms, fever, DIC, and an active MPS. Although each factor may function independently, it is much more likely that they interact, e.g., the hemolytic factors, fever, and/or DIC damage the erythrocyte membrane, which then more readily binds antigen–antibody complexes or complement, predisposing it to erythrophagocytosis.

During phases two and three, increased rates of hemolysis persist, and a progressive dyshemopoiesis develops. A possible explanation for the continuing erythrocyte destruction by the MPS in the absence of trypanosomes comes from the work of Jandl et al. (1965) who found that after prolonged and repeated stimulation the MPS in rats remains active long after the stimulant has been withdrawn. An analogous situation may exist in cattle in phases two to three and the MPS, no doubt stimulated initially by the massive trypanosome challenge, may continue to be active long after the disappearance of the parasite, thereby causing hypersequestration and increased red cell hemolysis.

In ferrokinetic studies carried out during phase two, there was no overt evidence of dyshemopoiesis, although the level of erythropoietic activity was surprisingly moderate in relation to the degree of anemia (Dargie et al. in press). Whereas there was accelerated utilization of transferrin-bound iron indicative of hyperactive marrow, the proportion of iron carried to the marrow and subsequently incorporated into erythrocytes was lower in infected cattle than in controls. Moreover, a substantial amount of the total iron incorporated into red cells was either reutilized extremely slowly or became unavailable for further hemoglobin synthesis following erythrophagocytosis. Either iron was being excreted or its release into plasma and subsequent transport to the bone marrow was being blocked by the MPS. The former seems unlikely because hemorrhage is not a major feature of the disease. Dargie et al. (in press) estimated that *T. congolense*-infected cattle lose between 40 and 55% of their circulatory iron. Their findings combined with the build up of hemosiderin deposits suggest defective iron reutilization and raise the possibility that, in long-standing infections, the marrow is effectively starved of iron and the anemia is complicated by dyshemopoiesis.

It seems the underlying mechanisms of anemia in phases two and three are similar to those in anemia associated with chronic disorders in humans (Karle 1974). The latter have been resolved by administration of testosterone, cobalt, or purified erythropoietin (Haurani and Green 1967; Zucker, Friedman, and Lysik 1974; Karle 1974), all of which may directly or indirectly release iron from the mononuclear phagocytic system, as well as acting directly on stem-cell proliferation. This offers the interesting possibility that their administration may be of therapeutic value in bovine trypanosomiasis.

Despite apparent elimination of the parasite from the blood and tissues during phase three, some cattle remain anemic although others do make a clinical recovery with a return of normal hematological values. In our studies, the trypanotolerant Ndama were most often able to recover. One possible explanation is that their MPS, following the disappearance of the parasite, returns to normal function more readily than does that of the Zebu because they sustain consistently lower parasitemias (Murray and Morrison in press) and, therefore, less MPS stimulation.

Death

Death in cattle due to African trypanosomiasis may occur at any time, depending on such factors as the animals' breed, previous exposure, and nutritional status as well as the level of challenge and the virulence of the organism. Our experience has been with cattle in natural conditions where

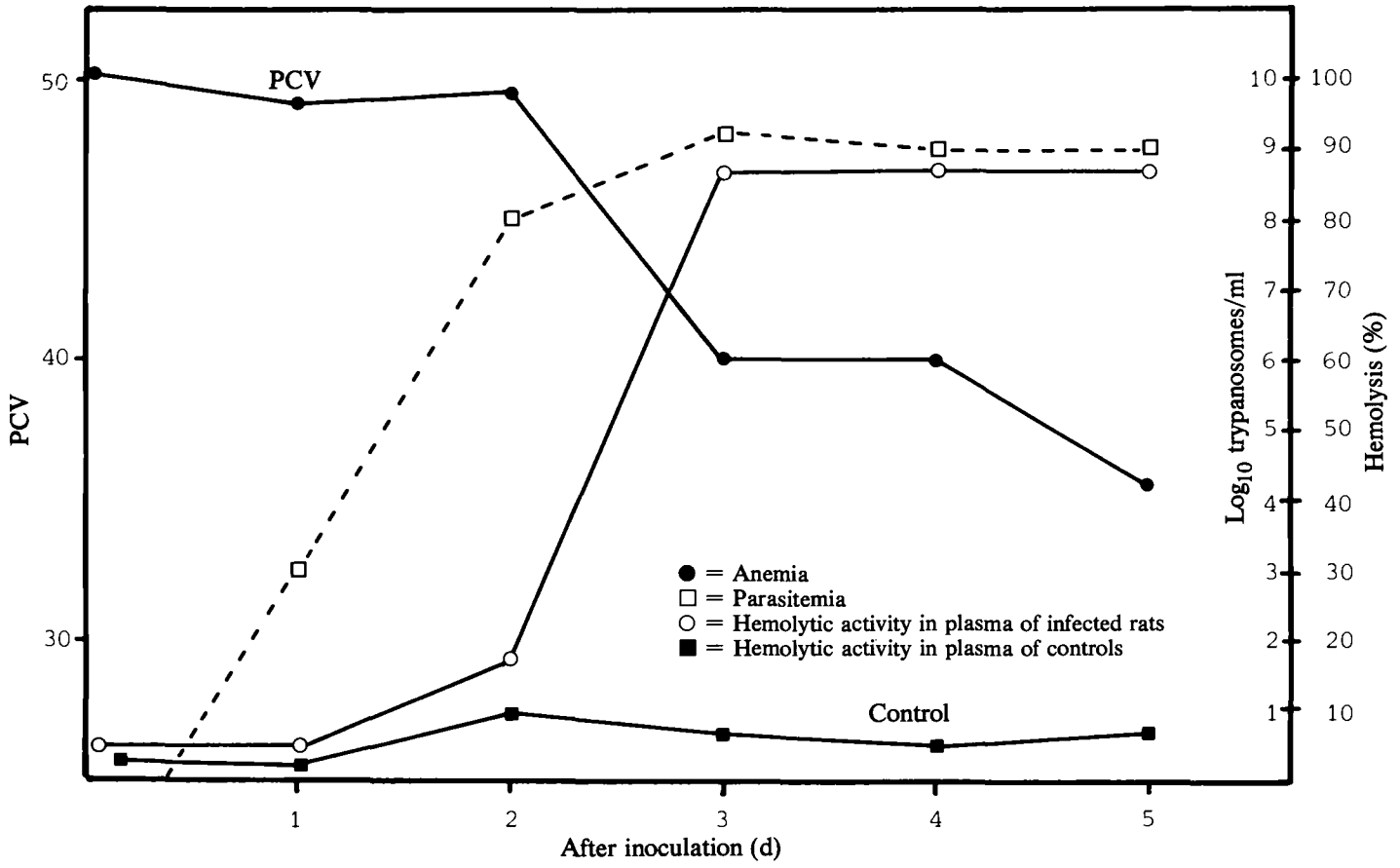

Fig. 2. *The development of anemia in rats infected with* T. brucei.

they sometimes forage up to 27 km in a day. In such circumstances, they often die of acute congestive heart failure from a combination of anemia, circulatory disturbance associated with increased vascular permeability, and myocardial damage (Murray, M. 1974; Murray, M. McIntyre, Murray et al. in press a, in press b). We have found severe myocardial damage in *T. congolense*-, *T. vivax*-, and *T. brucei*-infected cattle. We based our diagnosis on clinical signs correlated with postmortem findings. Terminally, cattle became extremely weak and lethargic, often refusing to rise. A jugular pulse was usually obvious and hemic murmurs were occasionally heard. Following tachycardia, often of a paroxysmal nature, bradycardia with a barely detectable pulse developed. Some animals, particularly those that survived for several days, developed subcutaneous edema in the submaxillary area, in the brisket, and in the lower abdomen. At necropsy, the heart was pale, dilated, and decompensated, and edema was a prominent feature in subcutaneous tissues and between skeletal muscle bundles. Often fluid was increased in the serosal cavities, especially in the pericardium and in the joints. Occasionally, there was edema of the folds of the abomasum and in the coils of the mesentery. The liver was swollen and sometimes mottled due to early chronic venous congestion. Considered together, these clinical and postmortem findings are characteristic of congestive heart failure.

Although a PCV of between 15 and 20% is unlikely to kill inactive cattle, similar PCV values in ranging animals may lead to death. An animal with a major red blood cell deficit is not able to forage over the wide distances required in the savanna, and its efforts to do so may cause cardiac decompensation. In this respect, we have frequently noted that animals in serious condition often improve dramatically if moved from the range and into a situation where they are fed and are not required to forage.

Conclusion

Throughout the course of trypanosomiasis, anemia persists as the cardinal sign, but its mechanisms and kinetics appear to vary, depending on the presence or absence of trypanosomes. Most research has concentrated on the early parasitemic phase when the anemia is hemolytic and red cell destruction is mainly by phagocytosis. We have directed special attention to the later phases when anemia persists even in the apparent absence of trypanosomes. The kinetics and mechanisms of this aspect of the disease are poorly understood, although there is evidence that dyshemopoiesis develops and hemolysis continues. The outcome of African trypanosomiasis in the bovine may be death, complete or partial recovery, or more commonly a syndrome characterized by stunting, wasting, and persistent low grade anemia, which we have termed chronic trypanosomiasis syndrome.

Erythropoietic response in bovine trypanosomiasis

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Abstract. The dynamics of red-cell destruction and production were measured using radio-tracer techniques in Ndama and Zebu cattle infected with *T. congolense* and in trypanosome-free animals of both breeds. The results showed that the primary cause of the anemia in infected animals was increased red-cell destruction due to excessive hemolysis. During the early phase of the disease, hemolysis is probably caused by the trypanosome, but the process may be potentiated by immunologic and physiological factors that render the cells more prone to erythrophagocytosis by an expanded mononuclear phagocytic system. The evidence suggests that in later stages of the disease excessive hemolysis continues even after trypanosomes have been eliminated from the blood and that it is due to the expanded and active mononuclear phagocytic system.

Although erythropoiesis was faster in infected animals, it was surprisingly moderate for the degree of anemia. In some cases, the poor response was due to iron deficiency, the reticuloendothelial system blocking the release of iron into the plasma. The effects of iron blockage were probably compounded by the animals' lack of appetite and low protein and iron intake. Calculations showed that, during the greatest drop in PCV values (i.e., 1–5 weeks post infection) the erythropoietic response was completely inadequate, possibly due to a time lag. Perhaps, an infected animal's PCV must fall to a certain threshold before more erythrocytes will be produced from stem-cell precursors.

The Ndama cattle were more resistant to *T. congolense* than were Zebu injected with the same number of parasites, but there was no evidence that the difference was in the ability to synthesize red cells. The difference may be that the Ndama impedes the parasite's ability to establish itself. The mechanism may be a genetically determined variation in immunologic responsiveness.

Several authors have claimed that the red-cell synthetic machinery is impaired in trypanosome-infected cattle (Fiennes 1970; Naylor 1971); others have indicated it to be normal or superior in laboratory animals (Boreham 1968a,b; Jennings et al. 1974; Assoku 1975; Holmes 1976); yet others have suggested that, in the bovine at least, erythropoiesis is accelerated at some stages and depressed at others (Wellde et al. 1972; Preston and Wellde 1976; MacKenzie et al. 1978). In few of these studies has erythropoiesis been quantified; therefore, it is difficult to assess the true situation.

The first attempt to assess the erythropoietic response quantitatively was made by Jennings et al. (1974) in rats infected with *T. brucei*; later, Mamo and Holmes (1975; Holmes 1976) studied calves and rabbits infected with *T. congolense*. The studies showed that infected animals experienced abnormally high rates of red-cell breakdown and attempted to compensate for it by increasing their rates of red-cell synthesis. In effect, there was nothing to indicate that erythropoiesis was suppressed. More recently, however, Preston and Wellde (1976), working with *T. congolense*-infected cattle and using sequential measurements, found red-cell synthesis to be enhanced during the early stages but suppressed in long-standing infections. The etiology of this phenomenon was not discussed, and the recorded changes were never related to the corresponding blood parasitemias or to the total circulating pools of red cells or hemoglobin.

Omissions such as these are numerous in the study of trypanosomiasis; one aspect in need of more detailed study is the phenomenon of trypanotolerance. What makes some breeds of livestock less susceptible than others to trypanosomiasis? Are they better able to resist the establishment or survival of trypanosomes, or are they better able to cope with the pathogenic effects

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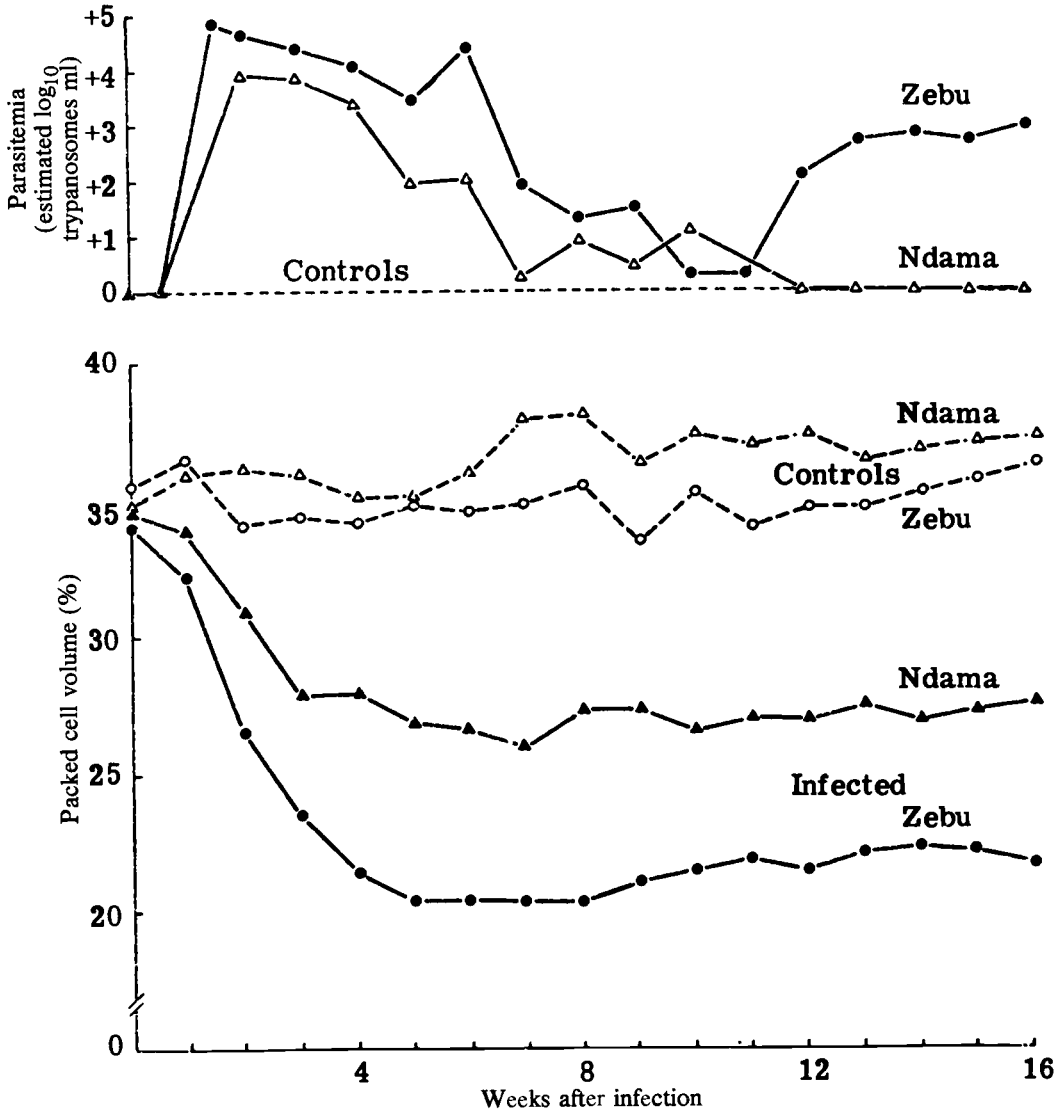

Fig. 1. *Parasitemia and PCV profiles of normal and* T. congolense*-infected Ndama and Zebu.*

once the parasites have become established? These aspects can only be examined by challenging different breeds of trypanosome-free animals with standard numbers of parasites, monitoring their subsequent patterns of parasitemia, and relating the findings to the accompanying anemia and red-cell kinetic patterns.

The work described in this presentation examined the kinetic aspects of the anemia in bovine trypanosomiasis by means of radioisotopic techniques; the aim was to obtain a better understanding of the etiology. Because the study was conducted using Ndama and Zebu cattle with no previous history of infection with trypanosomes, the data provide information on trypanotolerance and its basis.

Materials and Methods

The work was part of a large-scale study involving about 120 Ndama or Zebu cattle. The animals and the strain of *T. congolense* are described elsewhere (Murray et al. 1977). Seven cattle of each breed were individually inoculated

with 3.6×10^7 organisms and, 7 weeks later, along with three uninfected animals, were injected with radioisotope-labeled materials. Blood samples were collected daily for the next 2 months and were analyzed for radioactivity and the presence of parasites. A number of hematological parameters were also measured at intervals during the study.

Parasitemias were determined according to the buffy coat darkground phase techniques of Murray, Murray, and McIntyre (1977). The level of infection was graded from 1+ to 5+, a 5+ infection reflecting an estimated $\log_{10}$ trypanosomes/ml blood.

Standard solutions of isotopes — ^{125}I, ^{59}Fe, and ^{51}Cr — were used to label bovine albumin, transferrin, and autologous red cells. Full details of the labeling techniques are given elsewhere (Dargie et al. in press). The substances were injected via a jugular catheter, and blood samples collected at frequent intervals in the following 3 hours, twice daily during the next 6 days, and daily thereafter.

For radioactivity analyses, aliquots of plasma and blood were diluted with NaOH and counted in a well-type scintillation spectrometre. Isotopic mixtures were separated by means of spectrometry.

The methods used to calculate results are detailed in Dargie (1975) and Dargie et al. (in press). From the count rates of the samples collected and the relevant hematological indices, the parameters obtained were the plasma, circulating red cell, and blood volume; the survival of red cells in the circulation based on the half-lives of ^{51}Cr- and ^{59}Fe-labeled erythrocytes; and measurements of erythrocyte life span and red-cell synthesis based on measurements of plasma iron turnover and red-cell iron utilization.

Results

The packed-cell volume (PCV) levels of both infected groups deteriorated rapidly between the 1st and 6th weeks (Fig. 1). The values of the Ndama dropped by 25% from a preinfection mean of about 34% to a mean of 26%, whereas those of the Zebu fell by 41% from 34% to 20%. This difference was highly significant ($P<0.01$). Subsequently, the averages for both groups tended to increase slightly, but the Ndama maintained their superiority throughout ($P<0.02$). The PCVs of the controls fluctuated between 33% and 37%, and, although the Ndama generally had higher values, the difference was never significant. Red-cell counts and hemoglobin concentrations followed the pattern described for PCV.

The onset of anemia correlated closely with the appearance of parasites in the blood, and its severity with the level and duration of the initial peaks of parasitemia. Although the infections in both breeds became patent within 4 days of inoculation, the peak parasitemias were significantly lower and attained later in the Ndama; a peak of 4.0 ± 0.4 estimated $\log_{10}$ trypanosomes/ml was reached by day 13 in the Ndama, whereas a peak of 4.8 ± 0.2 was recorded by day 10 in the Zebu ($P<0.05$). The levels of parasitemia in the Ndama fell sharply between the 4th and 7th weeks to an estimated 0.5 $\log_{10}$ trypanosomes/ml, remained depressed until week 11, and then fell to 0, but those of the Zebu were elevated until week 6, and then declined progressively over the following 5 weeks to 0.5 $\log_{10}$/ml. Subsequently, all the animals came under a natural *Glossina palpalis gambiensis* challenge, the Zebu showing increased parasitemia levels and the Ndama exhibiting no effects.

The results of the blood volume measurements are given in my other report, p. 140. Not only are they valuable in examining the etiology of the anemia, but, combined with measurements of standard hematological parameters, e.g., the red-cell count and hemoglobin concentration, they enable calculation of an animal's total red cell, hemoglobin, and iron content (i.e., total red cell pool size = concentration × volume; and 3.4 mg

Table 1. Total circulating pools of red cells, hemoglobin, and iron during *T. congolense* infection in cattle.

		Preinfection			7 weeks post infection			15 weeks post infection		
Breed	Group	Red cells ($\times 10^{12}$)	Hb (g)	Fe (mg)	Red cells ($\times 10^{12}$)	Hb (g)	Fe (mg)	Red Cells ($\times 10^{12}$)	Hb (g)	Fe (mg)
Ndama	Infected	94	1737	5905	68	1250	4250	78	1444	4909
	Control	98	1814	6168	105	1937	6586	112	2074	7052
Zebu	Infected	108	1993	6776	51	975	3315	53	973	3309
	Control	107	1966	6684	112	2074	7052	119	2189	7442

iron/g hemoglobin) (Table 1). It was assumed throughout that total blood volume was 60 ml/kg in all animals.

The greater body weight of the Zebu before infection indicates larger blood volumes and greater reserves of all red-cell constituents (by about 10%) than in the Ndama. By the 7th week, however, the infected Zebu had lost 57×10^{12} red cells, about 1000 g hemoglobin, and nearly 3500 mg iron from their circulation; their Ndama counterparts had lost only 26×10^{12} red cells, about 490 g hemoglobin, and 1700 mg iron. These figures dramatically illustrate the relative insensitivity of PCV values for determining the severity of insult experienced by the two groups of animals, i.e., the difference in terms of PCV drop was only 30% (see Table 1, Dargie 1978), but in terms of total quantities of the constituents was almost 100%.

In effect, the Zebu were much more severely affected than is indicated by the breed PCV differences. By week 15 post infection, the total amounts of constituents in the Zebu were virtually identical to those at 8 weeks, but those of the Ndama had increased (by about 10×10^{12} red cells, 195 g hemoglobin, and 660 mg iron). Although peripheral blood concentrations were relatively steady in both groups, the differences between the two grew larger, albeit more slowly than early in infection.

The effect of *T. congolense* on rates of red-cell breakdown and synthesis and on iron utilization was measured between the 7th and 15th week of the study (Table 2). The ^{51}Cr red-cell half-life values demonstrate that both infected groups, but particularly the Zebu, experienced significantly faster rates of red-cell breakdown or loss from circulation than did the controls. The fact that PCV values were steady and total red-cell and hemoglobin pools increased slowly during this period is indirect evidence of increased erythropoiesis.

This was confirmed directly by ferrokinetic measurements: compared with the controls, the amount of iron carried from the plasma to the bone marrow (i.e., the plasma iron turnover rate, PITR) was increased on average by about threefold in the infected Zebu and twofold in the Ndama (Table 3). This was partly a reflection of the larger plasma iron pool of the infected animals but was mainly attributable to their greatly accelerated rates of plasma iron "clearance" (i.e., $t_{1/2}$). It should be emphasized that although the majority of infected animals had normal or elevated serum iron levels, total and latent iron-binding capacities, and serum transferrin concentrations (Table 3) two of the Zebu had extremely low values (e.g., serum iron levels < 100 μg/100 ml and TIBCs around 190 μg/100 ml). Significantly, such animals had total plasma iron pools of only 0.4 mg/kg and their PITRs were either comparable with or only slightly greater than the average control values. Both animals died before the experiment ended, their PCV values deteriorating rapidly (i.e., to around 9% and 12% at necropsy).

Although erythropoiesis generally proceeded at an accelerated rate in the infected animals, the response in a portion of cases was clearly suppressed. Because a proportion of the iron entering the marrow is not used in hemoglobin synthesis, the "effective" erythropoiesis is a reflection of the amount of red-cell iron actually incorporated in hemoglobin (Table 4). The results show that all the infected animals had speeds and absolute rates of incorporation significantly higher than did controls but the percent incorporation was significantly lower in the infected Zebu than in the Ndama. The time to maximum incorporation, however, was the same. Very significantly, there was a precipitous drop in the red-cell ^{59}Fe activity of both infected groups, and in the Zebu particularly, relative to the controls; thus, not only were the infected cattle losing red cells at an abnormally rapid rate, but they were also apparently unable to reutilize much of the constituent iron for hemoglobin synthesis.

Table 2. Red cell survival and plasma iron kinetics of cattle infected with *T. congolense* (± standard error).

Group	^{51}Cr red cell $t_{1/2}$ (d)	Plasma iron pool (ml/kg)	Plasma ^{59}Fe $t_{1/2}$ (min)	PITR (mg/kg/d)
Ndama				
Infected	9.3 ± 0.7	0.562 ± 0.035	79 ± 5.9	0.75 ± 0.09
Control	14.6 ± 0.5	0.474 ± 0.035	129 ± 20.6	0.36 ± 0.06
P value	<0.01	NS	<0.02	<0.05
Zebu				
Infected	8.3 ± 0.6	0.728 ± 0.072	73 ± 5.6	1.00 ± 0.09
Control	14.0 ± 0.4	0.475 ± 0.039	133 ± 7.5	0.36 ± 0.05
P value	<0.001	<0.05	<0.001	<0.002

Table 3. Serum iron and binding capacities of cattle 7 weeks after infection with *T. congolense* (±standard error).

Group	Serum iron (μg/100 ml)	TIBC (μg/100 ml)	LIBC (μg/100 ml)	Transferrin saturation (%)	Plasma transferrin (mg/100 ml)
Ndama					
Infected	120 ± 7.1	322 ± 12.3	202 ± 15.5	38 ± 3.0	258 ± 9.9
Control	122 ± 5.1	368 ± 21.7	246 ± 19.4	33 ± 1.7	295 ± 17.3
P value	NS	NS	NS	NS	NS
Zebu					
Infected	149 ± 13.2	355 ± 32.1	206 ± 22.4	43 ± 2.3	284 ± 25.9
Control	137 ± 7.8	438 ± 14.3	287 ± 2.0	31 ± 0.9	351 ± 11.6
P value	NS	NS	NS	<0.02	NS

The data given in tables 2 and 4 provide useful insights into the relative rates of hemoglobin catabolism (or loss) and regeneration. Because the total number of red cells and amounts of hemoglobin and iron represented by a particular "rate" of breakdown (i.e., by a given red-cell half-life or life span) depend on the prevailing pool sizes, the dynamic effects of trypanosomes on a host's red cells also depend on them. For example, in weeks 7–15 after infection, the controls had an average red-cell life span of about 80 days and a total red-cell pool varying between 100 and 110 × 10^{12} (tables 1 and 4); they were, therefore, catabolizing about 1.5 × 10^{12} cells, 26 g hemoglobin/day. In both groups presumably the same number of cells and amount of hemoglobin were synthesized. Conversion of the red-cell iron utilization data into total amounts gives a figure that corresponds very closely to the theoretical value (i.e., about 28 g). The small difference is explicable by the gradual increase in the hemoglobin pool found in association with growth (Table 1). In the infected Ndama, the red-cell life span was reduced to 34 days, but because of the lower pool size, this 250% increase corresponded to an absolute increase of only 27% (i.e., 1.9 × 10^{12} cells and 38 g hemoglobin). In the Zebu, where the life span was reduced to 18 days (i.e., indicative of a fourfold acceleration in red-cell breakdown), the number of red cells and amounts of hemoglobin catabolized were 3 × 10^{12} and 56 respectively (i.e., about twice normal). Because all the animals were roughly stable with respect to their total circulating pools, rates of synthesis must at least have been comparable to rates of breakdown, or even slightly greater in the case of the Ndama. The total number of red cells synthesized by the Ndama was only about 30% greater than their normal counterparts, whereas in the Zebu it was about twice normal. The figures recorded for red-cell utilization in the former suggest an absolute rate of hemoglobin synthesis of around 42 g/day, i.e., 4 g/day more than expected from the red-cell breakdown data or 224 g over the period of observation. About 195 g can be explained by the progressive increase in pool size. Figures for red-cell utilization indicate a total synthesis of 58 g/day, i.e., a figure corresponding very closely to that derived from the breakdown data.

Although the kinetics of the precipitous drop in

Table 4. Red cell iron kinetics and life span in cattle infected with *T. congolense* (± standard error).

Group	^{59}Fe utilization (%)	Maximum utilization (d)	Red-cell iron utilization (mg/kg/d)	^{59}Fe red-cell $t_{1/2}$ (d)	Red-cell life span (d)
Ndama					
Infected	88 ± 3.3	7.8 ± 0.9	0.66 ± 0.08	86.3 ± 8.4	34.3 ± 3.8
Control	91 ±7.0	18.3 ± 3.0	0.33 ± 0.06	198.0 ± 19.9	86.2 ± 15.9
P value	NS	<0.01	<0.05	<0.001	<0.01
Zebu					
Infected	75 ± 3.1	7.7 ± 0.6	0.80 ± 0.03	61.9 ± 6.0	17.8 ± 1.8
Control	90 ± 5.6	20.7 ± 1.8	0.32 ± 0.03	223.8 ± 20.0	72.7 ± 6.4
P value	<0.05	<0.001	<0.001	<0.001	<0.001

PCV values 1–4 weeks after infection cannot be gleaned from the data, the present study indicates that even without any increase in erythropoiesis, average losses of about 3.0×10^{12} erythrocytes/day would produce anemias of the severity recorded in the Ndama and Zebu between weeks 1 and 4, i.e., about 1.5 times greater than recorded later. If the ability of the bone marrow to increase its output increased, then the numbers involved would be even greater.

Discussion

Although most studies on the anemia of African trypanosomiasis have considered the disease a static rather than a dynamic condition, the factors involved probably vary during the disease, which frequently can be protracted. In the work reported here, the onset of anemia and the drop in PCV values correlated closely with the appearance, level, and duration of the initial parasitemia. However, even when parasites could no longer be detected in the blood, PCV values showed little tendency to recover, and subsequent peaks of parasitemia were not necessarily accompanied by further deterioration in PCVs.

The erythrokinetic studies of infected cattle demonstrated abnormally high rates of red-cell breakdown or loss between the 7th and 15th weeks. The two possible causes are hemorrhage and hemolysis. Because there was no evidence of increased fecal excretion of isotope in samples collected at random during the experiment, the former is unlikely. The signs pointed to the latter: the mononuclear phagocytic system (MPS) was manifestly expanded and active, and erythrophagocytosis was marked, particularly in the spleen, which was consistently enlarged (Murray, M., Huan, Lambert et al. in press).

There is now evidence that during the early parasitemic phases, red-cell destruction is triggered by the trypanosomes and that immunologic and physiologic factors render the cells more prone to erythrophagocytosis by an expanded MPS. For example, it has been demonstrated in vitro that African trypanosomes produce factors capable of damaging red blood cells (Huan et al. 1975; Tizard and Holmes 1976), and these have been found in the sera of trypanosome-infected cattle (Murray, M., Huan, Lambert et al. in press). Furthermore, complement and IgG and IgM immunoglobulins with antibody activity specific for *T. congolense* have been detected on red cells of infected calves (Kobayashi, Tizard, and Woo 1976). Finally, studies on the anemia associated with chronic disorders of humans have shown that small elevations in temperature cause increased osmotic fragility, accelerated hemolysis, and plasticity of red cells in vitro as well as a reduced red-cell life span and increased splenic stasis and destruction of erythrocytes in vivo (Karle 1968, 1974). In this study, pyrexia was a prominent sign in the cattle during parasitemic episodes and was likely linked to the anemia.

One finding of particular interest in the Ndama was the high rate of hemolysis that persisted even when trypanosomes were no longer detected in the blood. Although it is impossible to exclude the presence of small numbers of parasites or of antigens released after phagocytosis, it may be that the trypanosomes are not required for continued red-cell destruction after initial parasitemia. Histopathological studies of cattle showed that the MPS was expanded and may have been responsible for erythrocyte destruction. This possibility is supported by work in laboratory animals showing that if the MPS is expanded by nonspecific substances such as methyl cellulose, zymosan, or *Corynebacterium parvum*, increased red-cell destruction and anemia result (Gorstein and Benacerraf 1960; Nussenzweig 1967; Zuckerman, Abzug, and Burg 1969). If stimulated repeatedly, the MPS remains active long after the stimulant has been withdrawn (Jandl et al. 1965). It is possible that an analogous situation exists in cattle and that the MPS, no doubt stimulated initially by the massive trypanosome challenge, remains active after the disappearance of the parasite, thereby causing hypersequestration and increased red-cell hemolysis. The rates of plasma iron turnover and red-cell iron utilization indicate erythropoiesis was faster in the infected animals; therefore, there was no overt evidence of dyshemopoiesis. Nevertheless, the erythropoietic responses were surprisingly moderate for the degree of anemia and the number of cells being removed. The fact that sheep, swine, and humans can increase hemopoiesis by up to six times during parasitic or chemically induced anemias (Dargie 1975; Wintrobe 1967) implies that the ability of the animals to increase red-cell production was impaired.

The possible reasons are apparent in the iron metabolism data. Although the amount of iron carried to the bone marrow was increased, the rate of ^{59}Fe clearance from the plasma was slower than expected from the degree of anemia and was not greatly different in the Ndama and Zebu. Because erythropoiesis (Reissmann 1964) and plasma ^{59}Fe clearance rates (Berry and Dargie in press) are markedly reduced in the presence of a low protein intake, the relatively poor erythropoietic response

may partly reflect inappetence shown by the infected cattle and by the Zebu in particular (Dargie et al. in preparation). It is evident that within both infected groups, a proportion of the iron carried to the marrow was not incorporated into red cells. Thus, the effectiveness of increased utilization of transferrin-bound iron was limited by premature destruction of erythrocytes in the marrow and/or in the circulation. Of the total iron incorporated into red cells, a substantial proportion either was reutilized extremely slowly or became unavailable for further hemoglobin synthesis following erythrophagocytosis. This phenomenon has two possible explanations. Either the iron was excreted, which seems unlikely, or its release into the plasma and subsequent transport to the bone marrow was blocked by the reticuloendothelial system and the normal exchange between plasma and storage pools was disturbed. Whatever the cause, the change was obviously substantial because by the 7th week, the circulating iron pools of the infected Ndama and Zebu, i.e., total red-cell hemoglobin value $\times$ 3.4, were about 4000 mg and 3000 mg respectively compared to 6500 mg for the controls. As a consequence of their anemia, the infected cattle lost between 40 and 55% of their circulating iron. This loss, combined with the presence of massive hemosiderin deposits within the MPS, especially of the spleen, liver, bone marrow, and hemal nodes (Murray unpublished data), indicates defective iron utilization and raises the possibility that in severe or long-standing chronic infections, the marrow is effectively starved of iron and the anemia complicated by dyshemopoiesis. This theory is supported by a number of observations. First, the serum iron and transferrin concentrations and the plasma iron pool of the Zebu that died were only about half normal; their plasma iron turnover rates were comparable to the average for the controls; and their PCVs were deteriorating rapidly. Second, microcytosis (Fiennes 1970), hypoferremia (Tartour and Idris 1973), and low plasma iron turnover rates (Preston and Wellde 1976) attend the anemia of long-term cases. Third, in animals selected for necropsy, red pulp rarely constitutes more than 10–20% of longitudinal sections of femoral marrow and, in more advanced cases, is yellow and gelatinous, indicative of almost total unresponsiveness (Murray unpublished data). These findings suggest that the anemia is ultimately complicated by some degree of marrow dysfunction, the basis of which is probably reticuloendothelial iron blockage. A marrow dysfunction would explain the poor clinical response to trypanocidal therapy in animals with long-standing infections and would mean that the condition is morphologically, biochemically, and kinetically analogous to the anemias associated with chronic human disorders (reviewed by Cartwright and Lee 1975) and experimentally induced inflammation in laboratory rodents (Hershko, Cook, and Finch 1974). Testosterone and erythropoietin (Haurani and Green 1967; Zucker, Friedman, and Lysik 1974) have been used to resolve such anemias, and it may be that, in addition to their direct action on stem-cell proliferation, such hormones are capable, directly or indirectly, of releasing iron from the reticuloendothelial system.

Response Inadequate

In the work reported here, the erythropoietic response was completely inadequate to cope with the high rates of red-cell breakdown, which in all likelihood were associated with the early stages of the disease. Work on other parasitic infections has established that significant anemia consistently precedes the conversion of yellow to red marrow and that the erythropoietic system expands slowly in response to hemorrhagic stress (Ractliffe et al. 1969; Dargie and Allonby 1975; Berry and Dargie in press). If the same holds for trypanosomiasis, then the time lag, which is possibly the time taken by the host to reach a critical hematocrit value and to produce erythrocytes from stem-cell precursors, accelerates the development of the anemia and results in loss of large numbers of erythrocytes without significant replacement.

All the evidence from this study indicates that the Ndama's superior resistance to *T. congolense* infections lies in their ability to limit the level and duration of parasitemia. They do not seem to mount and maintain a more efficient erythropoietic response than do the Zebu, but they may be able to impede the process by which the parasite establishes itself, possibly through their inherent immunologic response. Desowitz (1959), investigating animals that had been previously exposed to trypanosomiasis, presented evidence that the Ndama controlled parasitemia through a secondary immune response that was superior to the Zebu's. The present study, which was carried out on cattle with no previous experience of trypanosomiasis, suggests that the Ndama has a better primary response. Whereas the genetic basis of differences in responsiveness among bovine breeds remains to be determined, work in mice may provide some insights: the ability of different inbred strains of mice to survive infections with *T. congolense* was based on their capacity to control levels of parasitemia and was inherited as a dominant trait (Morrison et al. 1978).

Pancytopenia in bovine trypanosomiasis

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Abstract. Sixty-eight cattle were experimentally infected with *T. congolense* TREU 112 (N = 18) or EATRO 1800 (25), or with *T. vivax* EATRO 1721 (25); all showed signs of pancytopenia, i.e., anemia, leukopenia, and thrombocytopenia. The *T. congolense*-induced anemia tended to be more persistent and to elicit a greater response than did the *T. vivax*-induced anemia. Trypanosome strain or species differences tended to be less important than age-related host differences.

Although anemia is a prominent sign of bovine trypanosomiasis, its causes are not fully established, and the pathogenesis of the *T. vivax*- and *T. congolense*-induced diseases is poorly understood. To further the understanding of anemia in bovine trypanosomiasis and to elucidate the pathogenesis of trypanosomiasis, investigators in Canada and in Kenya undertook complementary studies, the hematologic findings of which are compared herein.

Animals and Infection

In the first part of the Guelph study (Valli, Forsberg, and McSherry 1978), 12 of a group of 24 Holstein calves (group 1) were infected with 10^6 *T. congolense* TREU 112 and were used with their control group for a hematologic study of the development of anemia, including red blood cell life-span studies (^{51}Cr–RBC).

Of 12 additional calves (group 2), 6 were infected with *T. congolense* TREU 112 and were used for studies investigating the effects of bovine trypanosomiasis on blood coagulation.

In the second part of the Guelph study (Valli, Mills, and Lawson 1977), routine hematologic examination was conducted on 12 *T. congolense*-infected calves used to study biochemical changes and the attachment of *T. congolense* to endothelium. Neonatal and 6-month-old calves were used.

In the Kenya study (Maxie, Losos, and Tabel 1976, submitted for publication), 25 yearling Holstein–Freisian steers were experimentally infected with *T. vivax* (EATRO 1721), 25 with *T. congolense* (EATRO 1800), and 25 were controls.

Hematologic Findings

Erythron: The packed-cell volume (PCV), hemoglobin concentration, and red-cell count decreased by about 30% in all *T. congolense*- and *T. vivax*-infected cattle. About 2 weeks after infection, the animals reached the nadir of anemia. *T. congolense* caused a low-grade persistent anemia, but the PCV in the *T. vivax* group slowly returned toward normal.

The red-cell mean corpuscular volume (MCV) rose 30–50% in all infected groups to a peak at day 30–45 post infection: it then plateaued or slowly declined. This increase indicates the release of large, young red cells into circulation and is consistent with a normal responsive marrow. A prominent reticulocyte response was not evident in any infected group and, in fact, was not expected in the bovine species, given that the anemia in trypanosomiasis develops slowly and is not severe and that severe rapidly developing anemia is needed to produce a significant reticulocyte response.

The myeloid-to-erythroid ratio (M:E) determined from aspirates of sternal bone marrow decreased by about 60% in the *T. congolense*-infected calves and by about 30% in the *T. vivax*-infected calves. Thus, although both parasites caused the same degree of anemia, the *T. congolense* provoked a greater erythroid response. The increased marrow response was not sufficient to return the circulating

hemoglobin to normal levels in any of the animals. This finding indicates ongoing red-cell destruction, i.e., hemolysis. The neonatal calves were able to mount a greater erythroid response than were the more mature calves, as indicated by the increase in hematopoietic marrow noted at necropsy.

In summary, a mild-to-moderate hemolytic anemia developed slowly following the first parasitemic wave in both infections, and it elicited an appropriate erythroid response. The anemia was initially macrocytic and normochromic, and later tended to be normocytic, normochromic. Differences between *T. congolense* strains were not remarkable. The responsiveness of the marrow in the *T. vivax* calves was less than in the *T. congolense* calves and may reflect less severe ongoing red-cell destruction.

Leukon: A 30–50% decrease in the total leukocyte concentration occurred in all infected groups within about 1 week of infection. This moderate leukopenia was transient in the *T. vivax* group and was most prolonged in the *T. congolense*-infected calves studied in Kenya. Concomitant neutropenia and lymphopenia generally accounted for the leukopenia. Neutrophil concentrations decreased by 50–75% in the infected groups, whereas the lymphocyte count decreased by 25–40%. Lymphocytosis developed during chronic *T. congolense* infection in neonates in Guelph. Eosinopenia occurred in both infected groups in Kenya but not in Guelph. Changes in monocyte concentrations were not consistent or significant in any of the infections.

Platelet: A marked decrease of about 60% occurred in the platelet concentration in all infected groups during the 1st week of infection, and the lowest concentrations were encountered by 2 weeks' post infection. The platelet counts remained low in all infected groups throughout infection, implying either continued platelet consumption or decreased production.

Discussion

Both TREU 112 and EATRO 1800 isolates of *T. congolense* and EATRO 1721 *T. vivax* caused pancytopenia, i.e., anemia, leukopenia, and thrombocytopenia. The pancytopenia was closely associated with the first parasitemic wave. Similarities between the infections were more striking than were differences. The most notable differences were age-related in that the neonatal calves infected with *T. congolense* were more resistant to hematologic changes and were more capable of responding effectively than were older calves. This observation is in agreement with the relative age resistance noted by Fiennes et al. (1946).

The importance of pancytopenia in trypanosomiasis is open to debate. The anemia may be more important as a diagnostic criterion than as a disease factor. Because the anemia develops gradually and is often of moderate-to-mild severity, the animal's body compensates by drawing on mechanisms, such as its cardiopulmonary reserve. Heart rate and respiratory rate both increase, and homeostasis is maintained. By using reserve mechanisms at rest, the animal has less reserve available to respond to other increases in needs, so that its exercise tolerance may be decreased, a change that could be crucial for a grazing animal. The leukopenia seen in trypanosomiasis is no doubt important in lowering the host's defenses against other infectious agents and is probably directly involved in the increased susceptibility to secondary infection, because of decreased numbers of phagocytes and because of immunosuppression. Thrombocytopenia is probably an indicator of infection and may be due to pooling in, or premature removal of platelets by, an enlarged spleen. Another possibility is that it derives from the consumption of platelets during immune reactions wherein trypanosomal antigens adhere to platelets and lead to their aggregation. The thrombocytopenia is of moderate intensity and is not life-threatening unless a disseminated intravascular coagulation occurs.

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Effect of bovine trypanosomiasis on hematopoiesis

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Abstract. Bovine granulocyte/macrophage colonies ranging from 20 to 1000 cells were grown in methyl cellulose cultures. The percentage of adherent cells in bovine marrow was found to vary from 20 to 66%. Bovine erythroid colonies were cultured in plasma clots. Sera collected from cattle infected with *Trypanosoma vivax* or *T. congolense* inhibited the growth of granulocyte/macrophage colonies but not the erythroid colonies. Moreover, marrow collected from calves infected with *T. congolense* (TREU 112) showed a decreased efficiency of granulocyte macrophage colony formation in vitro. The future prospects of in vitro bone marrow culture as a tool for studying animal hematopoietic diseases are discussed.

Bradley and Metcalf (1966) in Australia and Pluznik and Sachs (1966) in Israel were the first to report the in vitro culture of mouse CFU-C colonies in agar. The progenitors of these colonies were designated "colony forming units in culture or CFU-C." Thereafter, the CFU-C colonies were successfully grown from human (Senn, McCulloch, and Till 1967), rat (Bradley and Siemienowicz 1968), bovine (Walker, Valli, and Lumsden 1974), and dog (Kovacs, Brunch, and Fliedner 1976) marrow cells. The growth of CFU-C colonies requires stimulation by colony stimulating activity (CSA), a heterogeneous group of glycoproteins. Price, McCulloch, and Till (1975) purified CSA from medium that had been conditioned by normal human peripheral leukocytes and observed that the CSA contained three molecular species of approximately 100000, 35000, and 15000 daltons. CSA has been produced from a variety of sources, e.g., from human peripheral leukocyte conditioned medium (Iscove et al. 1976; Price, McCulloch, and Till 1973), mouse kidney cells (Bradley and Summer 1968), human urine (Metcalf 1971; Stanley et al. 1977), animal postendotoxin injection sera (Bierman and Hood 1972; Chervenick 1972), and postirradiation sera (Beran 1975; Laissue et al. 1975). The principal source of CSA is the monocyte/macrophage type of cell (Chervenick and LoBuglio 1972; Golde, Cline, and Finley 1972), and endotoxin acts directly on this cell to stimulate CSA production (Goldman 1975). Price, McCulloch, and Till (1975) demonstrated that CSA from human peripheral blood leukocytes is a product of cell membranes.

In our experiments, bovine CFU-C cultures were prepared in methyl cellulose. Large colonies ranging in size from 20 to 1000 cells were produced within 6–7 days in culture. Bovine serum after endotoxin injection was used as the source of CSA. Our results confirmed that injection of animals with endotoxin increases serum levels of CSA significantly.

Messner, Till, and McCulloch (1973) observed that human marrow cell suspensions contain two different populations of cells, one population capable of adhering (adherent cells) and one incapable of adhering to glass and plastic (nonadherent cells). They observed that the adherent cells produce CSA and that their proportion in human marrow cells varies from 20 to 50%. In bovine marrow cells, we have found that the proportion of adherent cells varies from 20 to 60% and that there may be no marked difference between humans and cattle in the percentage of these cells. Moreover, no difference was observed between control and *T. vivax*-infected cattle in the percentage of adherent cells.

Erythroid (CFU-E) Colony Culture

Stephenson et al. (1971) were the first to report the in vitro culture of mouse erythroid colonies. They designated the cells originating these colonies as "erythroid colony forming units or CFU-E." They used a plasma clot technique, which was slightly modified later by McLeod, Shreeve, and Axelrad (1974) using microwells instead of petri dishes. The technique has since been successfully applied to human (Tepperman, Curtis, and McCulloch 1974), dog (Brown and Adamson 1977), and bovine (Kaaya, Valli, and Maxie 1978) marrow cells. CFU-E colonies have also been successfully grown in methyl cellulose using mouse (Hara and Ogawa 1977; Iscove and Sieber 1975) and human (Iscove, Sieber, and Winterhalter 1974; Ogawa et al. 1977) marrow cells. We have successfully grown bovine CFU-E colonies in plasma clots, using both the original method and the modified technique. However, we were unable to grow colonies in methyl cellulose cultures. Growth of bovine CFU-E in methyl cellulose appears to require culture conditions different from those described for mouse and human CFU-E.

Clinical Applications

The development of CFU-C culture technique by Bradley and Metcalf (1966) and Pluznik and Sachs (1966) and of CFU-E by Stephenson et al. (1971) opened a new era in clinical hematology. A few hematological diseases have already been studied using in vitro bone marrow culture, and useful results have been obtained, e.g., marrow from humans suffering from acute myelogenous leukemia has been found to be capable of forming very few small CFU-C colonies during relapse, and the efficiency of colony formation improves during remission (Harris and Freireitch 1970). Price, McCulloch, and Till (1975) observed that CSA from normal humans (peripheral leukocyte conditioned medium) contained three molecular types, i.e., 100 000, 35 000, and 15 000 daltons, but that CSA from leukemic patients in relapse contained only one molecular type, usually 35 000 daltons. In remission, leukemic patients exhibited all three types. Likewise, CSA from peripheral blood leukocytes of patients suffering from idiopathic acquired sideroblastic anemia, a disease that often develops into leukemia, contained only one molecular species (Till et al. 1975). These observations show that in vitro bone marrow culture may be a useful tool for the diagnosis of early relapses and completeness of remission and for predicting the prognosis of leukemias. The plasma clot culture has been used to study congenital hypoplastic anemia and pure red cell aplasia, and findings indicate that in congenital hypoplastic anemia, the inhibitor of erythropoiesis is located in the lymphocytes, whereas in pure red cell aplasia, the inhibitor is located in the serum (Hoffman et al. 1976). The standard agar culture for CFU-C has been used to detect inhibitors of granulopoiesis in sera of BALB/C mice (Metcalf and Russell 1976) and in dialysates of media conditioned by normal and leukemic mouse cells (Metcalf 1971) and to demonstrate the inhibitory effect of anti-mouse brain serum or mouse CFU-C colony formation in vitro (Meyer-Hamme and Bluestein 1978).

To date, in vitro bone marrow culture has not been used widely for studying animal diseases, but in future, it will likely be used for studying diseases associated with bone marrow depression anemias and myeloproliferative disorders.

In our experiments with in vitro bone marrow culture, we found that sera from *T. vivax*- and *T. congolense*-infected cattle inhibited granulopoiesis but not erythropoiesis (Table 1). The nature and source of the inhibitor are not yet known, but further work is being done to characterize it. Marrow collected from calves infected with *T. congolense* (TREU 112) shows a decreased efficiency of CFU-C colony formation (Table 2). Similar results were reported by Valli, Mills, and Lawson (1977). It is concluded that the stem-cell pool in the calves was reduced — a finding that suggests the inhibitor of leukopoiesis kills stem cells. We strongly believe that the leukopenia reported in cattle infected with *T. congolense*

Table 1. Effect of sera from *T. congolense*-infected and control calves on CFU-C culture (marrow was collected from a healthy calf and serum was collected 3 weeks post infection).

		Serum from control calf	
Cell concentrations		2×10^4	6×10^4
Colonies	dish 1	7	84
	dish 2	11	70
	dish 3	5	74
	dish 4	14	76
Mean per dish		9.25	76.00
		Serum from infected calf	
	dish 1	3	28
	dish 2	5	37
	dish 3	4	43
	dish 4	4	42
Mean per dish		4.00(S)[a]	37.50(S)[a]

[a]S = significant ($P < 0.05$).

Table 2. A comparison of CFU-C colonies from *T. congolense*-infected and control calf marrow samples (marrow was collected 3 weeks post infection).

Cell concentrations ($\times 10^4$)		0.5	0.8	1.0	2.0	4.0	6.0	8.0	10.0
		Control calf (no. 3)							
Colonies –	dish 1	0	6	40	104	226	497	537	682
	dish 2	4	10	32	125	250	442	601	786
	dish 3	3	5	23	132	168	337	454	751
	dish 4	2	7	20	108	282	400	417	708
Mean per dish		2.25	7.00	28.75	117.25	231.50	419.00	502.25	731.75
		Infected calf (no. 4)							
	dish 1	0	1	0	6	8	38	54	76
	dish 2	0	1	5	5	7	43	48	92
	dish 3	0	3	1	3	2	27	64	87
	dish 4	1	0	1	4	3	41	48	70
Mean per dish		0.25(NS)[a]	1.25(S)[a]	1.75(S)[a]	4.50(S)[a]	5.00(S)[a]	37.25(S)[a]	53.50(S)[a]	81.25(S)[a]

[a]S = significant ($P < 0.05$); NS = not significant.

(Losos et al. 1973; Naylor 1971) and *T. vivax* (Maxie, Losos, and Tabel 1976; Vohradsky 1971) results from loss of stem cells caused by circulating toxic factors. The fact that no inhibitor of erythropoiesis was detected in sera collected from our trypanosome-infected cattle does not confirm the absence of an erythropoietic inhibitor because the CFU-E is a much more mature progenitor than is CFU-C (Gregory, McCulloch, and Till 1973), and, therefore, the CFU-E might have differentiated beyond the inhibitable stage. It is also possible that the inhibitor, if present, is located in lymphocytes as is the case in congenital hypoplastic anemia (Hoffman et al. 1976).

Effects of *T. congolense* and *T. brucei* on the circulatory volumes of cattle

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Abstract. The plasma and circulating red-cell volumes of Ndama and Zebu cattle infected with *T. congolense* or *T. brucei* were measured simultaneously using, respectively, ^{125}I-labeled albumin and ^{51}Cr-labeled red cells and compared with figures for uninfected controls of each breed. Plasma volumes were also estimated using ^{59}Fe-labeled transferrin, and total blood volumes were calculated as the sum of plasma and red-cell volumes. Data from labeled albumin and red cells indicated that the plasma volumes of trypanosome-infected cattle were higher than normal and red-cell volumes were markedly reduced, netting no change in total blood volumes. The labeled transferrin showed plasma volumes significantly higher than those estimated with labeled albumin, suggesting hypervolemia and, hence, hemodilution in the anemia. However, I feel that ^{59}Fe-transferrin is unsuitable for measuring the plasma volume in animals and that, at present, there is no reliable evidence implicating hemodilution in the anemia accompanying early and acute trypanosomiasis. However, the process may be involved during later stages.

The chemical make-up of blood obtained from animals infected with trypanosomes often differs substantially from that of normal animals. The differences vary with the host–parasite system and with the duration and severity of the infection but invariably include three major features, i.e., anemia, hypoalbuminemia, and hyperglobulinemia (see Fiennes 1970). Consequently, measuring the PCV, hemoglobin, plasma albumin, and globulin concentration is the accepted method of assessing the infection and judging trypanotolerance (Roberts and Gray 1973). But are they valid criteria?

The answer is no if changes in "concentration" are a manifestation of changes in "total amount." Measurements of concentration depend as much on the volume of the body compartment sampled (i.e., the blood or plasma), as on the rates of synthesis and catabolism and must be accompanied by measurements of compartment volumes to provide an accurate picture of trypanosomiasis, especially the anemia, which is the major clinical feature of the disease.

Recently, authors have claimed that hemodilution is important in the etiology of anemia in trypanosomiasis. Naylor (1971) described increases of up to 30% in the plasma volumes of cattle infected with *T. congolense*, and using the same host–parasite system, Holmes (1976) showed that severely anemic calves (i.e., with PCVs of 17%) had normal total red cell volumes but markedly expanded plasma and, hence, total blood volumes. Both studies implied that the anemia was not due to any reduction in the total number of red cells in the circulation but rather to abnormal fluid retention within the plasma compartment. Holmes (1976) also reported hypervolemia due to plasma volume expansion in rabbits undergoing *T. congolense* infections, although in this species, red cell volumes were markedly reduced. Likewise, Anosa and Isoun (1976), working with sheep and goats, concluded that hypervolemia associated with increased plasma and reduced red cell volumes was a feature of *T. vivax* infections, although Clarkson (1968) showed in similar infections that plasma volumes were expanded but total blood volumes were unaltered due to decreases in the number of circulating red cells. Clarkson's finding was supported by Jennings et al. (1974) on the basis of studies in rodents infected with *T. brucei*.

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Clearly, information about the changes in blood compartment volumes and their role, if any, in the anemia and other clinical manifestations of trypanosomiasis is sparse and inconsistent. For this reason, my colleagues and I conducted a detailed study in which we compared the blood volumes of normal and infected cattle using radioisotopic methods. The results suggest that hemodilution is not involved in the anemia.

Materials and Methods

We used 20 4-year-old Ndama and 20 4-year-old Zebu cattle, subcutaneously inoculating 7 animals of each breed with 3.6×10^7 *T. congolense* and a further 7 with 2.5×10^8 *T. brucei*. Twelve animals of both breeds acted as controls. The animals and parasites have been described previously (Murray, P.K., Murray, Wallace et al. in press a). All the animals were injected simultaneously with ^{125}I-labeled albumin, ^{59}Fe-labeled transferrin, and autologous red cells labeled with ^{51}Cr, 7 weeks after the trypanosome inoculations. Full details of the labeling techniques and of the ancillary hematological measurements are given elsewhere (Dargie et al. in press, in preparation).

All volumes were estimated on the basis of the isotope dilution technique (International Atomic Energy Agency 1974). After intravenous injection of the labeled substances, we waited 15 minutes for equilibration and then collected a blood sample from the opposite vein. Subsequently, we took samples every 15 minutes for 3 hours and assayed them for radioactivity. We estimated plasma volumes by dividing the total radioactivity injected by the ^{125}I-activity in 1 ml of each plasma sample. Results were compared with estimates based on ^{59}Fe-transferrin, which we obtained by extrapolating the plasma-^{59}Fe counts during the first 3 hours to zero and using the result (rate/ml) to divide into the total ^{59}Fe-activity injected. Circulating red cell volumes were measured with the labeled red cells by dividing the total injected ^{51}Cr-activity by the activity apparent in the whole blood sample corrected for PCV. Blood volume was calculated as the sum of plasma and red cell volume, and all volumes were then related to body weight data.

Any leakage of labeled substances from the bloodstream during equilibration erroneously raises the values for plasma and, hence, blood volume. To eliminate this possibility in our studies, we measured the transcapillary exchange rate of albumin (TER_{alb}). The plasma ^{125}I count rates were plotted against time, and the resulting graph was used to calculate the half-life ($t_{1/2}$) of the labeled albumin and, thereby, TER_{alb} (Parving and Gyntelberg 1973):

$$TER_{alb}\,(\%/h) = \frac{0.693}{t_{1/2}\,(h)}$$

Results

The results (tables 1 and 2) show that irrespective of the breed of animal and species of trypanosome, the plasma volumes of infected cattle were higher and their circulating red cell volumes lower than the corresponding control values. The changes were more pronounced in the Zebu cattle and in the

Table 1. Blood compartment volumes of normal cattle and cattle infected with *T. congolense* (Mean ± SD).

Group	PCV (%)	Plasma[a] (ml/kg)	Red cells (ml/kg)	Blood[b] (ml/kg)	Plasma[c] (ml/kg)	Blood[d] (ml/kg)
Ndama						
Infected	27 ± 2.4	46.9 ± 5.6	15.6 ± 2.1	62.5 ± 5.6	58.7 ± 4.0[e]	74.3 ± 5.8[e]
Control	38 ± 2.4	38.8 ± 3.4	21.4 ± 3.0	60.2 ± 3.4	42.5 ± 3.6	63.9 ± 6.2
P value	<0.001	<0.02	<0.01	NS	<0.001	<0.001
Zebu						
Infected	19 ± 2.9	48.7 ± 5.3	10.9 ± 2.9	59.6 ± 7.7	71.0 ± 10.6[e]	81.9 ± 13.1[e]
Control	35 ± 3.4	34.4 ± 2.0	20.8 ± 4.2	55.2 ± 4.4	36.7 ± 2.2	57.5 ± 7.6
P value	<0.001	<0.002	<0.002	NS	<0.001	<0.001

[a] Measured with ^{125}I-albumin.
[b] Calculated as sum of plasma volume (measured with labeled albumin) and red cell volume (measured with ^{51}Cr-labeled red cells).
[c] Measured with ^{59}Fe-transferrin.
[d] Calculated from ^{59}Fe and ^{51}Cr data.
[e] Significantly greater than corresponding ^{125}I data.

Table 2. Blood compartment volumes of normal cattle and cattle infected with *T. brucei* (Mean ± SD).

Group	PCV (%)	Plasma[a] (ml/kg)	Red cells (ml/kg)	Blood[b] (ml/kg)	Plasma[c] (ml/kg)	Blood[d] (ml/kg)
Ndama						
Infected	27 ± 3.3	44.3 ± 8.7	16.6 ± 3.4	60.9 ± 9.1	53.2 ± 12.4[e]	69.8 ± 13.6[e]
Control	35 ± 2.6	38.7 ± 6.4	20.8 ± 2.7	59.5 ± 5.8	40.6 ± 8.6	61.4 ±9.8
P value	<0.01	NS	NS	NS	NS	NS
Zebu						
Infected	24 ± 4.1	47.2 ± 10.0	14.4 ± 2.6	61.6 ± 8.7	58.7 ± 11.4[e]	73.1 ± 14.2[e]
Control	33 ± 3.8	40.3 ± 3.2	18.5 ± 1.1	58.8 ± 7.0	43.8 ± 5.7	62.3 ± 8.0
P value	<0.02	NS	<0.05	NS	<0.05	<0.02

[a] Measured with ^{125}I-albumin.
[b] Calculated as the sum of plasma volume (estimated with labeled albumin) and red cell volume (measured with ^{51}Cr-labeled red cells).
[c] Measured with ^{59}Fe-transferrin.
[d] Calculated from ^{59}Fe and ^{51}Cr data.
[e] Significantly higher than corresponding ^{125}I data.

animals infected with *T. congolense*, but in no case were total blood volumes as measured with labeled albumin and red cells altered by infection. On the other hand, estimates of plasma volume based on ^{59}Fe-labeled transferrin were manifestly higher than those obtained from the same animals using labeled albumin. The differences appeared to depend on the hematological status of the animals, ranging from less than 5% in the controls to about 45% in the Zebu infected with *T. congolense*. Calculations of total blood volume based on labeled transferrin and red cells indicated that infected animals experienced some hypervolemia and, hence, hemodilution.

Measurements of the transcapillary exchange rate of albumin (Table 3) revealed no significant differences between infected and control groups. Also, in all animals less than 16% of the injected

Table 3. Half-lives of ^{125}I-albumin and ^{59}Fe-transferrin in normal and trypanosome-infected cattle.

Group	^{125}I-albumin $t_{1/2}$ (h)	^{59}Fe-transferrin $t_{1/2}$ (h)	$TER_{(alb)}$ (%/h)	^{59}Fe removal (%/h)
Ndama				
Infected				
(T. congolense)	17.5 ±1.94	1.32 ±0.09	3.97	52.5
Control	16.8 ±2.02	2.15 ±0.34	4.12	32.2
P value	NS	<0.02	NS	<0.02
Zebu				
Infected				
(T. congolense)	20.3 ± 2.36	1.22 ± 0.09	3.41	56.8
Control	21.6 ± 1.81	2.22 ± 0.13	3.21	31.2
P value	NS	<0.001	NS	<0.001
Ndama				
Infected				
(T. brucei)	19.6 ± 1.25	1.93 ± 0.10	3.53	35.9
Control	23.2 ±2.78	2.40 ±0.19	2.99	28.9
P value	NS	NS	NS	NS
Zebu				
Infected				
(T. brucei)	24.6 ± 2.67	1.50 ± 0.16	2.82	46.2
Control	20.8 ± 1.95	2.35 ± 0.27	3.33	29.5
P value	NS	<0.05	NS	<0.02

radioactivity had escaped from the circulation during the first 3 hours following inoculation. The behaviour of labeled transferrin during the same period differed markedly in infected and control cattle. The ^{59}Fe half-life values were significantly shorter in the infected animals, and particularly in those infected with *T. congolense*. Also noteworthy in all animals was the plasma clearance of ^{59}Fe, which was much faster than clearance of ^{125}I. The difference ranged from about 10 times in the controls to more than 15 times in the Zebu infected with *T. congolense*.

Discussion

The data obtained from labeled albumin and red cells provided no evidence that hemodilution was involved in producing the anemia exhibited by the infected cattle. Nor was there any evidence that capillary permeability was altered by infection. Admittedly, the plasma volumes of affected cattle were higher than normal but the increases were balanced by equally dramatic reductions in the size of the red cell compartment, and the total blood volumes were unaltered. However, when labeled transferrin was employed as the plasma diluent, the resulting plasma volumes were significantly higher than those obtained with labeled albumin, and hypervolemia and, hence, hemodilution were implicated. Also this label disappeared much faster from the circulation of the infected cattle than from controls — a result that suggests increased capillary permeability.

Isotope measurements of any body compartment are accurate only if the labeled substance equilibrates throughout the compartment and is not lost along the way. Also, during equilibration, the labeled substance must behave identically in animals that are to be compared. Our results show that labeled albumin conformed to both criteria, but ^{59}Fe-transferrin conformed to neither. For example, the half-life of the former was virtually identical in all groups, and only a small proportion of the total injected activity had disappeared from the circulation by 3 hours. On the other hand, the half-life of labeled transferrin was much shorter in the infected cattle, and by 3 hours after injection only a small proportion of original activity remained in the circulation.

Such anomalies are explicable by the contrasting metabolic behaviour of the two labels; when injected into the bloodstream, ^{59}Fe is carried by transferrin to the bone marrow for incorporation into red cell precursors; consequently, even in normal animals, this label probably never equilibrates fully within the plasma compartment, the "equilibrium" activity is reduced, and the plasma volume overestimated; moreover its rate of disappearance is not an index of capillary permeability. In animals whose demand for red cells is greater than normal, e.g, those infected with trypanosomes, equilibration is even less complete, and the overestimation of plasma volume even more serious. Labeled albumin enters the interstitial fluid slowly and only after 4–5 days equilibrates fully with the total exchangeable albumin pool. Because its rate of disappearance during the early post-injection period is slow, it can be used with confidence both to estimate plasma volume and to assess capillary permeability. Thus, there was no evidence during the early stages of infection that the anemia was due to anything other than a drop in the total number of red cells in their circulation. Neither was there any indication of increased vascular permeability. The data demonstrate clearly that labeled transferrin is unsuitable for measuring plasma and hence total blood volume in animals with anemia; it is also unsuitable for plasma volume comparisons between normal and infected cattle. ^{59}Fe was employed as the plasma diluent in the studies of Holmes (1976); therefore the finding of hypervolemia and hemodilution in the anemia was not valid. Naylor's (1971) conclusion was also invalid because no measurements were made of red cell volume; and the results of Anosa and Isoun (1976) are suspect because different animals were used for the plasma and red cell volume measurements. Our findings demonstrate an increase in plasma volume compatible only with the drop in red cell volume and agree with the earlier results of Clarkson (1968); both studies used labeled albumin to estimate plasma volume. In other words, at present, there is no reliable evidence that hemodilution contributes to the anemia of trypanosomiasis in its early and acute stages in the bovine.

The changes in total red cell volumes of infected cattle are beyond the scope of this paper but the accompanying expansions in plasma volume are noteworthy. Rapid loss of red cells from the circulation has important consequences that, if not counteracted quickly, prove fatal. Most important is the inevitable drop in arterial blood pressure, hypotension, resulting on the one hand from lowered blood viscosity and peripheral resistance, and on the other from reduced blood volume, venous return, and hence cardiac output. The bovine has compensatory mechanisms to restore blood pressure and thereby preclude possible ischemia of the brain and cardiac muscle. Two mechanisms are particularly important. First, is the movement of interstitial fluid into the vascular

system to counteract decreased blood volume. Second is alteration in the activity of baroreceptors within the carotid sinus, aorta, etc. Baroreceptors respond to a drop in blood pressure by slowing their rate of discharge. The result is that heart rate and contractility increase and the arterioles and veins constrict. All these events contribute toward restoring blood pressure and maintaining cardiovascular function.

Movement of fluid from the interstitium into the circulatory space (and vice versa) occurs at the capillaries, and two opposing forces are operative, i.e., the hydrostatic pressure difference between capillary and interstitial fluid pressures and the water concentration difference between plasma and interstitial fluid. The loss of red cells from the circulation decreases blood volume and pressure and increases arteriolar constriction. As a result, the capillary hydrostatic pressure is reduced. This decrease causes interstitial fluid to enter the plasma and thereby increase the plasma volume and restore the blood volume to normal. Hence, expansions of plasma volume such as we observed are normal responses to threatened hypovolemia and circulatory collapse and not abnormal accumulations of fluid within the circulatory space. They result in some dilution of hemoglobin and plasma proteins, but their importance should be put in perspective. For example, in the animals infected with *T. congolense*, if the plasma volume had failed to expand to maintain the total blood volume, the average PCVs would have been 20% as opposed to 19%, not a sufficient difference to contribute markedly to the pathogenesis of the anemia. Hemodilution could contribute meaningfully to pathogenesis only if the plasma volume increased out of all proportion to the attendant drop in red cell volume. Although this was not observed here and there is no reliable evidence for it in trypanosomiasis, some features of the disease suggest that hemodilution in some hosts or at particular stages has a bearing on the condition. For example, plasma volume expands disproportionately in situations involving disturbances in plasma protein metabolism. Excessive production of IgG in myelomatosis or of IgM in Waldestrom's macroglobulinemia are often associated with high plasma volumes, and a parallel situation can be produced experimentally by hyperimmunization (Birke 1966). The increase in colloid osmotic pressure precipitates the volume change. A comparable event may occur in trypanosomiasis, where large amounts of immunoglobulin and particularly IgM (see Luckins 1972) appear in the circulation. In my experience, however, these increases are rarely sufficient to produce significant hyperproteinemia in cattle. Rather, in most cases, albumin synthesis is lower than normal, and the total amount of albumin within the circulation is either unaltered or reduced (Dargie et al. in preparation). Hence, despite pronounced hyperglobulinemia, the colloid osmotic pressure does not seem to increase enough to produce the hypervolemias claimed by some authors.

Plasma volume also expands in situations where primary disturbances in plasma protein metabolism are unlikely, e.g., in splenomegaly (Crane, Wells, and Jones 1974) and hepatomegaly (Lieberman and Reynolds 1967). Splenomegaly is a common finding in animal trypanosomiasis, especially in laboratory rodents (Sadun et al. 1973; Losos and Ikede 1972; Murray, M. 1974), and may be responsible for reports of hemodilution. Splanchnic blood flow and volume may be raised, and portal hypertension may exist. The resultant trapping of blood in the splanchnic circulation would reduce the so-called effective blood volume, stimulate the renin-angiotensin-aldosterone system, promote sodium and water retention by the kidneys, and expand the extracellular volume.

Another possibility relates to the condition of the heart in trypanosomiasis. In rodents infected with *T. brucei* and, to a lesser extent, cattle infected with *T. congolense* (Murray, M. 1974), myocarditis may be severe. Congestive heart failure may be an important element in the pathogenesis of the disease, the accompanying decrease in cardiac contractility and output and failure of normal sodium and water excretion by the kidneys resulting in fluid retention and engorgement of the veins and capillaries. This, combined with the invariably severe concomitant hypoalbuminemia (Dargie et al. in preparation) may well account for the edematous condition of many infected animals.

Hemodilution in bovine trypanosomiasis

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Abstract. Accurate estimation of total blood volume requires independent, simultaneous measurements of red cell mass and plasma volume. These studies have not been conducted conclusively in cattle, and the occurrence of hemodilution in bovine trypanosomiasis remains to be proved.

Anemia is a prominent finding in bovine trypanosomiasis, and although it is generally recognized as being hemolytic in origin (Jennings 1976), it may also be a reflection of hemodilution (Fiennes 1954; Naylor 1971) or of bone marrow depression (Naylor 1971). Anemia can reliably be estimated by packed cell volume (PCV) only if the blood volume is normal. Hemodilution is the process by which the blood volume is expanded, usually through increased plasma volume, causing dilution of red cells. If the peripheral blood PCV is used to measure anemia, during hemodilution, the PCV estimate of red cell mass will be erroneously low (Fig. 1). The converse of hemodilution is hemoconcentration, in which the blood volume is reduced due to fluid loss, e.g., dehydration due to diarrhea; PCV in hemoconcentration is falsely elevated. In other words, the PCV is a good estimate of the red-cell mass only if the blood volume is normal.

Blood Volume Determination

The blood volume is maintained within a narrow range even in the face of wide fluctuations in PCV, because the plasma volume rapidly compensates for changes in red cell mass (Schalm, Jain, and Carroll 1975; Wintrobe et al. 1975). Estimation of blood volume is most accurately made by independent, simultaneous estimates of red cell mass and plasma volume; extrapolating blood volume from plasma volume plus hematocrit measurements is inaccurate unless the ratio of body hematocrit-to-venous hematocrit (BH/VH) is known. The body hematocrit is usually overestimated by the venous hematocrit by 10% because of the increased concentration of red cells in large veins, such as the jugulars. The BH/VH ratio, or F cells ratio, is normally about 1:1.1 but can vary in disease. The ratio increases in cases of splenomegaly, due to RBC pooling in the spleen, and decreases during edema (Fudenberg et al. 1961). Thus, particularly in disease states, independent measures of red cell and plasma volumes are required.

Plasma volume can be reasonably accurately determined by dye dilution (T-1824, Evan's blue) or by isotope dilution (radio-labeled protein, e.g ^{131}I-albumin). Red cell mass is usually measured by dilution of red cells, which have been labeled with ^{51}Cr either in vitro or in vivo.

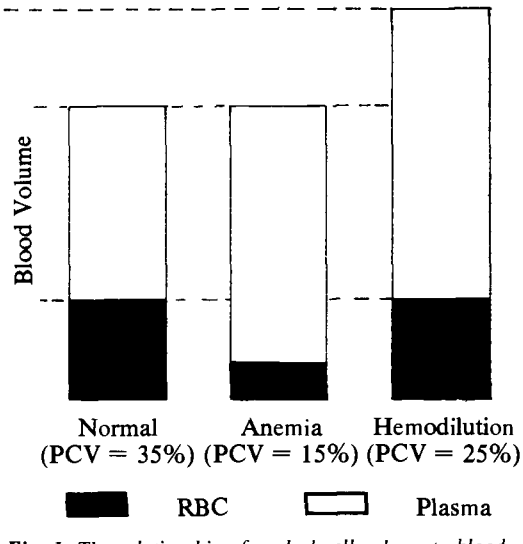

Fig. 1. The relationship of packed cell volume to blood volume.

Hemodilution in Trypanosomiasis

Fiennes (1954) reported the development of hydremia, or hemodilution, at day 6 post infection in one *T. congolense*-infected ox, determined by vital red dilution.

Rees and Clarkson (1967) showed, by Evan's blue dilution, a 16% increase in the plasma volumes of *T. vivax*-infected sheep. In their study, "total blood volume of the infected animals did not differ from the controls and cell volume (ml/kg) and packed cell volume (%) were significantly reduced."

Clarkson (1968) reported an 18% increase in the plasma volumes of *T. vivax*-infected sheep based on Evan's blue dilutions, but there was no change in the total blood volume. He suggested that the increased gamma globulin may act as a plasma-volume expander. Clarkson has since been cited as having shown the existence of hemodilution in trypanosomiasis, e.g., by Holmes and Jennings (1976).

Naylor (1971) determined, by using Evan's blue dye dilution, that the plasma volume in *T. congolense*-infected cattle increased by one-third during the first 6 weeks of infection. The packed cell volumes apparently decreased from about 36% to about 20% over this period. Although in his work "no measurement of blood volume was made," he concluded that "a relative deficiency of blood cells occurs initially due to hemodilution," even though he had no proof. As with Clarkson (1968), Naylor has been cited as having demonstrated the occurrence of hemodilution.

In a radioisotopic study (^{51}Cr, ^{59}Fe), Mamo and Holmes (1975) demonstrated that, in Zebu cattle chronically infected with *T. congolense*, severe anemia was due primarily to loss of red cells from the circulation. The blood volumes of their cattle 12–16 weeks post infection were not significantly different from controls. Thus, they did not detect hemodilution in their cattle.

Mamo and Holmes (1975) reported that, in *T. congolense*-infected Zebu cattle, "as the anemia developed, there was an absolute fall in the circulating red cell mass and an over-compensatory increase in the plasma volume. This suggests that the hemodilution effect was of secondary importance in bringing about the anemia." Details of their study were not given in their short note.

Anosa and Isoun (1976) measured plasma volumes with ^{131}I-albumin and red cell volumes with ^{51}Cr-RBC in *T. vivax*-infected sheep and goats. They showed significant increases in blood volume and plasma volume, with a concomitant decrease in red cell mass. They concluded that the anemia was "attributable partly to hemodilution and partly to an actual decrease in total volume of circulating red blood cells." Their report appears to be the first adequately documented proof of the occurrence of hemodilution in trypanosomiasis.

Holmes and Jennings (1975) demonstrated increased plasma volumes (^{59}Fe transferrin) and decreased red cell mass (^{51}Cr-RBC) in both *T. congolense*- and *T. brucei*-infected rabbits. Blood volumes appear to have been increased. These changes were noted as early as 1 week post infection, and the volumes rapidly returned to normal 1 week following treatment. They commented that the increase in plasma volume is real and probably due to peripheral vasodilation and is not an artifact due to leakage of labeled protein from the circulation.

In the above studies, which have used independent measurements of plasma and red cell volumes to obtain the blood volume, hemodilution has been shown to occur only in *T. vivax*-infected sheep and goats (Anosa and Isoun 1976) and in rabbits infected with *T. congolense* or *T. brucei* (Holmes and Jennings 1975). Hemodilution remains to be demonstrated in bovine trypanosomiasis.

Materials and Methods

In Guelph, Canada, measurements of hemodilution were carried out as part of other work on red cell life span as determined with ^{51}Cr labeling of red cells and with kinetic iron studies where transferrin was labeled with ^{59}Fe.

Determinations of red cell mass were carried out on 24 Holstein calves of which 13 were infected with 1×10^6 *T. congolense* TREU 112 intravenously at age 3–5 months, and 11 calves were controls (Valli, Forsberg, and McSherry 1978). Thirty millilitres of blood were anticoagulated with citrate; the plasma was removed by centrifugation, and 250–300 μCi of sodium chromate ^{51}Cr were added and incubated for 25 minutes at 37 °C. Labeled red cells were then washed three times in saline and injected intravenously. Post-injection samples were taken at 10 and 30 minutes on each calf. Red cell mass was calculated directly and blood volume indirectly, based on the hematocrit value.

Measurements of plasma volume were carried out in two groups of 12 Holstein calves each. One group were neonatal calves of which six were given 2.8×10^7 *T. congolense* TREU 112 organisms intravenously, and the second group of calves aged 5–6 months were given 8.5×10^7 organisms intravenously (Valli, Mills, and Lawson 1977). In

Table 1. Body compartment volumes determined in control, *T. vivax*- and *T. congolense*-infected Zebu calves.

Calf	Infection	Day	PCV (%)	wt (kg)	RBC mass (RCM, ml/kg)	Plasma volume (PV, ml/kg)	Blood volume (RCM + PV, ml/kg)	Extracellular fluid volume (ml/kg)	Total body water (ml/kg)
469	Control	–	36	202	23.1	34.5	57.6	238	648
543	*T. vivax*	80	30	195	21.0	36.3	57.3	210	623
568	*T. congolense*	80	14	143	7.1	54.3	61.4	335	576

[a] Dilutions of radioisotopes were used to determine red-cell mass (^{51}Cr-RBC), plasma volume (^{125}I-HSA), extracellular fluid volume (^{35}S), and total body water (^{3}HOH).

both experiments the organisms had been passaged through rats immediately before infection. Each group of calves used for plasma volume determinations was studied on two occasions during the 2nd and 6th week of infection along with the respective controls. Transferrin labeling was accomplished by incubating 6 ml of heparinized plasma with 50 μCi ^{59}Fe for the larger calves and 30 μCi ^{59}Fe for the neonatal calves, followed by incubation at 37 °C for 30 minutes or at room temperature overnight. Each labeled sample was then injected intravenously into the donor calves and post-injection samples were collected at 3, 6, 10, 15, and 30 minutes from each calf. Plasma volumes were determined directly and red-cell mass was determined indirectly.

In Kenya, a pilot experiment was carried out to characterize the erythron and body fluid compartments simultaneously. These parameters were determined as follows: red-cell mass (^{51}Cr-RBC, 2 μCi/kg), plasma volume (^{125}I-human serum albumin, 0.1 μCi/kg), extracellular fluid volume (^{35}S, 1 μCi/kg), and total body water (^{3}HOH, 1 μCi/kg), by methods similar to those of Bauer, Willis, Burt, and Grim (1975). Radioisotopes were obtained from the Radiochemical Centre Ltd., Amersham, England. Three Boran *(Bos indicus)* steers were used in the study. One steer had been infected with *T. vivax* (EATRO 1721), one with *T. congolense* (EATRO 1800), and one was an uninfected control.

Results

In the Guelph study, 15 determinations were carried out over 18 weeks. The total blood volume of infected calves was significantly higher than that of control calves during the 6th, 11th, 13th, and 17th weeks of observation. The plasma volumes determined indirectly were significantly higher in infected calves during the 2nd, 3rd, 4th, 5th, 7th, 11th, 13th, 16th, 17th, and 18th weeks of observation. Most significantly, the red cell mass determined directly (^{51}Cr-RBC) was significantly lower than that of the controls during the 4th and 11th weeks only.

In the younger calves after 2 weeks of infection, the plasma volume in control calves was 52 ± 4 (mean ± 95% confidence interval) ml per kg body weight, whereas in infected calves the plasma volume was 68 ± 13 ml per kg. These differences were significant. At day 14, the red cell mass in infected and control calves was not significantly different. At the 6th week of infection, the young control calves had plasma volume 47 ± 2 ml/kg body weight compared to 64 ± 2.5 ml/kg in infected calves. These results were significantly different; the red cell mass, however, in infected calves was not significantly different from control calves. In other words, plasma volumes were significantly increased in these calves at 2 and 6 weeks post infection.

Plasma volume determinations on the 6-month-old calves at the 2nd week of infection were 44 ± 5 ml/kg body weight in control calves and 58 ± 6 ml/kg in infected calves, and these results were significantly different. The red cell mass in infected animals was not different from that in controls. At the 6th week of infection in 6-month-old calves, the plasma volume was not determined; the red cell mass on the day of iron administration was 24 ± 3 ml/kg in control calves and 14 ± 4 ml in infected calves, the results being significantly different.

In the Kenya study, 80 days post infection, the *T. vivax*-infected calf had a PCV of 30%, and its red cell mass, plasma volume, blood volume, extracellular fluid volume, and total body water were essentially the same as those of the control calf (Table 1). However, the *T. congolense*-infected calf was anemic (PCV = 14%), had one-third of the normal red cell mass, a 50% increase in its plasma volume, and an essentially normal blood volume (Table 1). The extracellular fluid volume (i.e., plasma volume + interstitial fluid) of this calf was about 50% greater than normal, whereas the total body water was some-

what decreased, indicating decreased intracellular fluid (Table 1). Only the pilot studies were completed on erythrokinetics and body fluid compartments.

Convincing evidence of the occurrence of hemodilution in trypanosomiasis has only recently appeared (Anosa and Isoun 1976; Holmes and Jennings 1976). The studies conducted at Guelph and reported herein indicate that plasma volume expansion occurred in *T. congolense*-infected calves and that hemodilution may therefore have contributed to the diagnosis of anemia. However, independent measurements of plasma and red cell volumes were not made in the calves, and the blood volumes may be inaccurate. The findings from the preliminary study in Kenya do not indicate the presence of hemodilution in chronic *T. vivax* or *T. congolense* infection in cattle. The question of hemodilution in bovine trypanosomiasis thus remains unanswered.

Discussion summary

J.D. Dargie and P.A. D'Alesandro

The hematopoietic tissue responses in bovine trypanosomiasis are both complex and variable and are influenced by many factors including the breed, sex, age, and nutritional status of the infected animal as well as by the number and species of the trypanosomes. Much emphasis is placed on the anemia and pancytopenia that accompany trypanosomiasis and on the mechanisms by which they come about, but other manifestations of the disease, e.g., the changes in blood volumes, capillary permeability, and heart function have attracted considerable interest.

There are three basic but overlapping stages in the anemia of bovine African trypanosomiases. The initial phase corresponds to the first parasitemic peak, during which PCV values drop markedly, splenomegaly occurs, erythrocytes are destroyed rapidly, and there is an active — though limited — erythropoietic response. A hemolytic toxin, causing early red cell destruction, is produced by dying trypanosomes and is heat stable and trypsin-sensitive. It has been shown to cause red cell hemolysis in vitro and, most significantly, in vivo in rats. This material is not the free fatty acid produced by autolyzed trypanosomes and described by Tizard et al. p.103, nor is it likely to be the variant-specific glycoprotein described by Cross p.32, because its molecular weight is only about 12 000 daltons. And although it may be related to immunologic mechanisms, such as adsorbed antigen–antibody complexes that render the erythrocytes more prone to erythrophagocytosis in the expanded and active MPS in the spleen, the toxin is more likely an inductive agent that irreversibly damages the red cell surface and/or potentiates adherence of immune complexes to red cells. The role of fever in promoting red cell destruction should not be underestimated, however, and, like the other factors, should be further investigated.

The number of erythrocytes being destroyed correlates well with the levels of parasitemia and may have to reach a critical threshold before awakening an erythropoietic response. Early in the disease, the erythropoietic response is poor but age dependent in that young animals respond better than their older counterparts and have a lower susceptibility to trypanosomiasis (see Wellde et al. p. 82). During the first phase, the animals respond well to trypanocidal therapy.

Phase two, described as the chronic phase of the anemia, occasions little or no further drop in PCV values; but infected animals are stunted, the spleen size is variable, and there is marked hemosiderosis, particularly of the spleen, liver, and bone marrow. Parasitemia levels vary but are usually low. The animals do not respond or respond very poorly to trypanocidal therapy. Results indicate that red cell breakdown is persistently increased during this period, even in the apparent absence of parasites in the circulation, but that affected animals nevertheless respond through an increased, albeit limited, marrow response. Infected animals

also experience a degree of reticuloendothelial iron blockage, and in a proportion of cases the resultant iron deficiency leads to depressed erythropoiesis. Clearly, further work should be conducted to ascertain how such large and apparently unavailable iron deposits might be unlocked and utilized by the bone marrow.

The final phase of the anemia is usually associated with complete or partial recovery; however, a proportion of cases (i.e., 30% of Ndama and 60% surviving Zebu cattle) do not recover. During this period, the spleen is no longer enlarged, but the MPS remains active. There is continued erythrophagocytosis and hemosiderosis, and the bone marrow becomes yellow and gelatinous. Parasitemia levels are low and intermittent.

There is insufficient evidence that antigen–antibody complexes play a significant role in the anemia, although they are demonstrable on red cells. Studies on the survival of red cells in vivo must be conducted before this point can be resolved satisfactorily.

Along with the anemia, there are marked changes in other bone marrow-derived cells in trypanosome-infected animals. Pancytopenia is an important feature of the disease and is probably associated with suppressed production of cells, although the possibility of increased consumption cannot be excluded. Pancytopenia, and particularly neutropenia, may predispose animals to secondary infections, e.g., salmonellosis. Thrombocytopenia is a constant manifestation of trypanosomiasis in the bovine and persists throughout the entire course of the disease, developing most rapidly in the early stages.

Serum from animals infected with *T. congolense* and *T. vivax* has been shown in vitro (bone marrow culture) to inhibit granulocyte, but not erythroid colony formation — a finding that focuses attention on the effects of trypanosomes on cell division and maturation. In many diseases, e.g., tumours, babesiosis, and malaria, the "target cell" seems to be nonspecific inhibition of the division of rapidly dividing cells, and agents, such as BCG, *Corynebacterium parvum*, and endotoxin, produce similar effects. It may be that the trypanosome acts as a similar target cell and that the side effects are inhibition of cell division in the marrow and destruction of sperm cells.

Hemodilution, although postulated as a feature of trypanosomiasis, has not been demonstrated, although it may occur during the terminal stages of the disease. The degree of anemia is clearly related to the drop in total red cell mass and hence PCV. Whereas all animals do experience an increase in plasma volume, it only accounts for space left by the destroyed red cells. In animals infected with *T. congolense*, extracellular and interstitial fluid volumes are increased, but total body water is not. Thus, reduced intracellular water is likely, and, hence, also reduced cell mass. Much more work is required to establish how trypanosomes affect fluid and electrolyte balance and distribution, as well as body weight; in future studies, investigators must keep in mind that the disease in feed-lot animals is probably quite different from that in range animals and that much more emphasis should be placed on the latter.

Methods of measuring the effects of the disease need to be critically analyzed. For instance, labeled albumin may not produce a true picture of the capillary bed. Using it, Dargie p. 140 could find no evidence of increased capillary permeability in animals infected for 8 weeks with *T. congolense* or *T. brucei*, and yet, as indicated by Murray p. 121, Boreham p. 114, and Zwart and Veenendaal p. 111, there is obvious evidence of edema. In the bovine, it is most noticeable during congestive heart failure. At present, despite the large number of clinical, biochemical, and hematological manifestations of trypanosomiasis, the PCV is probably the most practical indicator of the severity of the disease.

Serum protein changes in bovine trypanosomiasis: a review

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Abstract. Changes in serum levels of total protein, albumin, complement, IgG, IgG1, IgG2, IgM, and heterophile antibodies of infected cattle are reported. It appears that the most striking serum change associated with trypanosomiasis in cattle is a pronounced and persistent hypocomplementemia.

Most studies on serum proteins in bovine trypanosomiasis have dealt with their levels during infection; hardly any information is available on their metabolic kinetics. To date, the reports have been entirely descriptive and provide only limited insight into how serum protein levels affect the whole pathophysiology of a disease. As long as rate of synthesis and rate of catabolism are unknown, interpretation of such data is restricted and often speculative.

I am going to report on serum levels of total protein, albumin, complement, IgG, IgG1, IgG2, IgM, and heterophile antibodies. My data derive from a study in which G.J. Losos, M.G. Maxie, and I experimentally infected Holstein cattle with either *T. vivax* EATRO 1721 or *T. congolense* EATRO 1800.

There are conflicting reports on serum levels of total protein. Decreased levels were reported by Fiennes, Jones, and Laws (1946) in calves infected with *T. congolense* and by Anosa and Isoun (1976) in sheep infected with *T. vivax*. Clarkson (1968) found normal levels in sheep infected with *T. vivax*, whereas Anosa and Isoun (1976) reported enhanced serum levels in three goats infected with *T. vivax*. We found significantly decreased serum protein levels in calves infected with *T. congolense* (66% of control values) and *T. vivax* (71% of control values) (Tabel, Losos, and Maxie in prepartion a).

Albumin

In previous studies, albumin levels have been found to be decreased in sheep infected with *T. vivax* (Anosa and Isoun 1976; Clarkson 1968), although Anosa and Isoun (1976) reported enhanced levels in three goats infected with *T. vivax*. At this conference, Dargie and Valli reported decreased serum albumin levels in cattle with trypanosomiasis, and we observed a significant drop of serum albumin at 15 days post infection in cattle infected with *T. vivax* or *T. congolense* (Tabel, Losos, and Maxie in preparation a). Plasma dilution may account for the changes in serum albumin concentrations, but a decreased rate of synthesis or an increased rate of catabolism cannot be disregarded as a potential cause.

Complement

Complement component C3 has been found to be decreased in the serum of patients with sleeping sickness (Greenwood and Whittle 1976b), in monkeys infected with *T. brucei rhodesiense* (Nagle et al. 1974), in rats infected with *T. lewisi* (Jarvinen and Dalmasso 1976), and in cattle infected with *T. congolense* (Kobayashi and Tizard 1976). Clarkson, Penhale, and McKenna (1975) reported decreased serum levels of a "7S γ1" protein in calves infected with *T. vivax*, and although they did not identify the protein, they described its precipitation pattern in immunoelectrophoresis, strongly implicating C3.

In our studies, we observed, a threefold drop of serum concentrations of C3 in cattle infected with either *T. vivax* or *T. congolense* (Tabel, Losos, and

Maxie in preparation a). The levels began to drop with the peak and decline of the first wave of parasitemia. In the *T. vivax*-infected animals, decreases in C3 levels correlated with the distinct first and second wave of parasitemia. We also observed a decline of total hemolytic complement activity in both infections; the initial drop again was associated with the end of the first wave of parasitemia (Tabel, Losos, and Maxie in preparation a). In *T. vivax* infections, the hemolytic complement levels were on average reduced to 5% of the control values, and at day 17, the mean hemolytic complement level dropped to 2% of the control levels. Hemolytic complement activity did not return to normal during the observation, which was about 70 days. In *T. congolense* infections, the hemolytic complement levels were, on average, reduced to 20% of the control levels and, like those in *T. vivax*, did not return to normal. It has been reported that hemolytic serum complement returns to almost normal levels within 6 days of treatment with Berenil (Rurangirwa unpublished data).

To my knowledge, there is no other infectious disease with such a pronounced and persistent hypocomplementemia. Together with neutropenia, it probably contributes to the enhanced susceptibility to secondary infections frequently observed in trypanosomiasis (Mott 1906; Peruzzi 1928; Parkin and Hornby 1930; Hull 1971; Maxie, Losos, and Tabel submitted for publication). At present, it is unknown whether decreases in complement activity and serum C3 levels are partially caused by decreased synthesis of complement components. However, immune complexes presumably consume considerable complement in combatting the repeated parasitemias. Another mechanism of complement consumption is the activation of complement by trypanosomal material like surface glycoprotein (Musoke and Barbet 1977; Nielsen and Sheppard 1977; Nielsen, Sheppard, Tizard et al. 1978b). The activation of complement indicates generation of complement-split products. The release of the anaphylatoxins C3a and C5a may cause dilation and may enhance permeability of capillaries (Müller-Eberhard 1968), accounting for the widespread edema observed in cattle (Maxie, Losos, and Tabel submitted for publication).

Hypocomplementemia potentially deranges the immune system, as shown in studies on decomplementation of mice and chickens. Pepys (1972, 1974, 1976) and Nielsen and White (1974; White and Nielsen 1975) have found that decomplementation results in immunosuppression or modulation of the immune response, and Rurangirwa, Tabel, and Losos p. 165 have shown that immunosuppression plays a part in bovine trypanosomiasis.

Immunoglobulins

IgG: Desowitz (1959) reported increases of IgG in Ndama cattle naturally infected with trypanosomes, and Luckins (1972) found a twofold increase of serum levels of IgG in Zebu cattle naturally infected. But Clarkson, Penhale, and McKenna (1975) noted that levels of IgG were little increased in calves experimentally infected with *T. vivax*. There is no evidence in bovine trypanosomiasis of enhanced IgG synthesis due to polyclonal activation of B cells as has been observed in *T. brucei*-infected mice (Hudson et al. 1976).

IgG1: Luckins (1976) and Nielsen, Sheppard, Tizard et al. (in press) reported normal serum IgG1 levels in calves experimentally infected with *T. congolense*, but Kobayashi and Tizard (1976) found rising serum IgG1 levels (2.2 times original levels) up to the 7th week in calves infected with *T. congolense*. In the Kobayashi–Tizard study, the serum levels subsequently dropped to normal. In contrast, we observed a transient decrease of serum IgG1 in *T. vivax*-infected cattle and a drop to about 70% of control values in cattle infected with *T. congolense* (Tabel, Losos, and Maxie in preparation b).

IgG2: In calves experimentally infected with *T. congolense* TREU 112, Nielsen, Sheppard, Tizard et al. (in press) found normal values for serum IgG2, whereas Kobayashi and Tizard (1976) reported little but steady increases to about 1.5 times the original values at 18 weeks post infection. Luckins (1972) observed normal serum IgG2 levels in Zebu cattle infected with *T. congolense*, whereas we found serum IgG2 levels fluctuating in *T. vivax*-infected cattle more than in controls but remaining within normal range (Tabel, Losos, and Maxie in preparation b). In our cattle experimentally infected with *T. congolense*, the serum IgG2 increased steadily to 1.5 times the control values at 7 weeks post infection.

IgM: Elevated serum levels of IgM have been found fairly consistently in humans with sleeping sickness (Mattern et al. 1961; Masseyeff, Blondel, and Mattern 1972; Mannweiler, Geister, and Krampitz 1969) and in laboratory animals infected with *T. brucei* subspecies (Seed et al. 1969; Capbern, Mattern, and Pautrizel 1974; Hudson et al. 1976; Campbell, Esser, and Weinbaum 1977). Moulton and Sollod (1976) noted that calves experimentally infected with *T. brucei* had a twofold increase of serum IgM. Serum IgM has also been found to be increased in natural infections

of cattle (Gidel and Leporte 1962; Bideau, Gidel, and Moity 1966; Luckins 1972), Luckins (1972) reporting increases of up to nine times the normal levels in Zebu cattle. In cattle experimentally infected with *T. vivax*, IgM levels were elevated up to four times (Clarkson, Penhale, and McKenna 1975; Luckins 1976). In cattle infected with *T. vivax*, we observed a decline of total serum IgM concentration from day 4 to day 9 post infection, although complement-fixing antibodies against *T. vivax* were rising. The drop was associated with the decline of the first wave of parasitemia. Subsequently, mean serum IgM levels rose to 1.5 times the normal values (Tabel, Losos, and Maxie in preparation b). The picture is somewhat different with experimental infections of *T. congolense*. Some infected calves have had 24-fold increases of serum IgM (Kobayashi and Tizard 1976), whereas others have been found to have normal concentrations (Nielsen, Sheppard, Tizard et al. in press). Luckins (1976) reported a decline of serum IgM to subnormal levels after an initial rise in two Zebu cattle experimentally infected with *T. congolense*. We found mean values of serum IgM concentrations fluctuating but remaining within normal ranges in cattle infected with *T. congolense* (Tabel, Losos, and Maxie in preparation b). In our studies, some animals periodically had elevated serum IgM, whereas others had marked decreased concentrations.

Calves infected with *T. vivax* have demonstrated increased heterophile antibodies to erythrocytes of chicken and sheep (Clarkson, Penhale, and McKenna 1975; Tabel, Losos, and Maxie in preparation b). We found the profile of serum levels of heterophile antibodies very much resembles the profile obtained with IgM. We also found decreased levels of antibodies to chicken erythrocytes at day 9 post infection. In *T. congolense*-infected cattle, heterophile antibodies were not elevated.

Elevated serum IgM in trypanosomiasis of monkeys has been shown to be due to synthesis of immunoglobulin that has no antibody activity to trypanosomes (Houba, Brown, and Allison 1969). There is evidence that some homogenates of trypanosomes contain a mitogen for B lymphocytes (Esuruoso 1976; Mansfield, Craig, and Stelzer 1976; Corsini et al. 1977) and cause a polyclonal synthesis of immunoglobulins; however *T. congolense* homogenates lack mitogenic activity for lymphocytes of mice, guinea pigs, and rats (Mansfield, Craig, and Stelzer 1976). This observation is in accordance with the absence of markedly elevated serum IgM levels in cattle infected with *T. congolense* observed by Luckins (1976) and also by us (Tabel, Losos, and Maxie in preparation b). The moderately increased levels of serum IgM in *T. vivax* infection in cattle may be satisfactorily explained by elevated levels of antibodies to *T. vivax* antigens.

It is not clear what causes the marked drop of serum IgM levels in cattle at 9 days post infection. Adsorption of serum IgM to *T. vivax* organisms has been ruled out because no serum proteins were demonstrated on *T. vivax* organisms isolated from cattle (Tabel unpublished data). It is more likely that the synthesis of all IgM other than anti-*T. vivax* antibody was suppressed. Immunosuppression has been demonstrated in *T. brucei*-infected mice as early as 2 days post infection (Jayawardena and Waksman 1977).

Lymphoid changes in African trypanosomiasis

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Abstract. Using standard histological techniques and immunofluorescence, we studied changes in the lymphoid organs of C3H/He mice experimentally infected with *T. congolense*; we compared them with those that occur in the lymphoid organs of trypanosome-infected cattle.

In infected mice, splenic cellular changes are considered to occur in two phases. First is an early proliferative phase, which is coincident with the first peak of parasitemia and is characterized by widespread hyperplasia of the white pulp with production of large numbers of Ig-containing cells and expansion of the erythropoietic component of the red pulp. This is followed by a more protracted phase during which there is a gradual cellular depletion of the white pulp and further expansion of the red pulp due to increased numbers of erythropoietic cells and, to a lesser extent, granulopoietic cells and macrophages. During infection, well-developed germinal centres are rarely observed in the spleen. Changes in the lymph nodes are less severe and occur later than in the spleen. There is marked proliferative activity, particularly in the follicular cortex, and large numbers of plasma cells are present in the medullary cords. In long-standing infections, there is some depletion of lymph node cortices but this is not as severe as in the spleen.

In cattle, marked proliferative activity occurs in the spleen and lymph nodes during the first 3–4 months of infection, but it differs from that found in mice: there is little disorganization of the lymphoid architecture, and widespread, active germinal centres are found. At this time, there does not appear to be lymphoid depletion, although we have some evidence that it does occur in long-standing infections.

From a number of studies carried out in recent years, it is clear that, in laboratory animals infected with African trypanosomes, severe functional changes occur in the immune system. The responses of spleen cells from affected animals have been shown to be profoundly suppressed in a wide range of in vitro T- and B-cell functional assays (reviewed by Pearson et al. in press). At the same time, infected animals show delayed rejection of allogeneic skin grafts (Pearson et al. 1978). However, despite the severe depression, large amounts of immunoglobulin are produced resulting in hypergammaglobulinemia (Askonas et al. in press; Morrison unpublished data). Although these functional changes are well documented, only a few attempts have been made to correlate them with cellular and architectural changes in the microenvironment of the lymphoid organs (Murray, M. et al. 1974; Murray 1974). In the present paper we describe the microscopic changes in the lymphoid organs of mice infected with *T. congolense* and consider their potential functional relevance. In addition, changes in the lymphoid organs of trypanosome-infected cattle are briefly discussed in so far as they differ from those found in the mouse.

The isolate of *T. congolense* that we have used produces long-standing infection in many inbred strains of mice (Morrison et al. 1978). We have studied C3H/He mice, the majority of which die 30–70 days after infection. In these animals parasitemia is first detectable about 6 days after infection and reaches an initial peak on day 8 or 9 (Fig. 1). After falling to undetectable levels for 1–2 days the parasitemia then increases and remains at a high level.

In our initial studies in *T. congolense*-infected mice, it was obvious that the changes observed in the spleen were much more pronounced and occurred much earlier than in other lymphoid organs. This is probably a consequence of the fact that *T. congolense* is a strictly intravascular parasite. Thus, we concentrated our investigations on the spleen.

The white pulp of the normal spleen is composed of several distinct compartments (Fig. 2). A cuff of small lymphocytes known as the periarteriolar lymphatic sheath (PALS) is found surrounding the splenic arterioles. The majority of the lymphocytes in this area are derived from the thymus (T-dependent) and are negative for surface immunoglobulin (Ig) (Fig. 3). Outwith the PALS are found a series of follicles that are populated predominantly by surface Ig positive bone marrow-derived (B-dependent) lymphocytes (Fig. 3). Upon antigenic challenge, germinal centres containing numerous lymphoblasts, develop within the follicles. Surrounding both the PALS and the follicles is the marginal zone. This is made up of a fine network of reticular fibres and contains numerous phagocytic cells and lymphocytes, most of which are positive for surface Ig. The open component of the splenic blood circulation involves the passage of blood from the central arterioles via capillaries into the marginal zone. Thus, the marginal zone is the point of entry into the spleen of circulating lymphocytes and antigens, and it is thought that the phagocytic cells in this area are important in antigen-trapping. Lymphocytes that enter the marginal zone may either pass into the red pulp or migrate into the white pulp.

To assess the cellular changes that occur in the spleen and to enumerate them, we carried out a preliminary experiment in which we examined stained cytospin preparation of spleen cell suspensions from normal and infected mice at intervals up

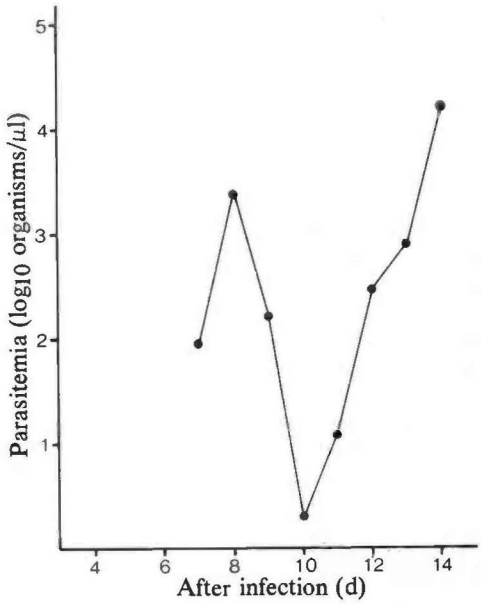

Fig. 1. Daily mean values for parasitemia in a group of 12 C3H/He mice infected with T. congolense.

to 35 days after infection. During this period there was a 10–12-fold increase in total spleen cellularity in infected mice. Early after infection, a peak of proliferative activity occurred coincident with the first peak of parasitemia; at this time there was a marked increase in plasma cells and a corresponding decrease in the percentage of small and medium

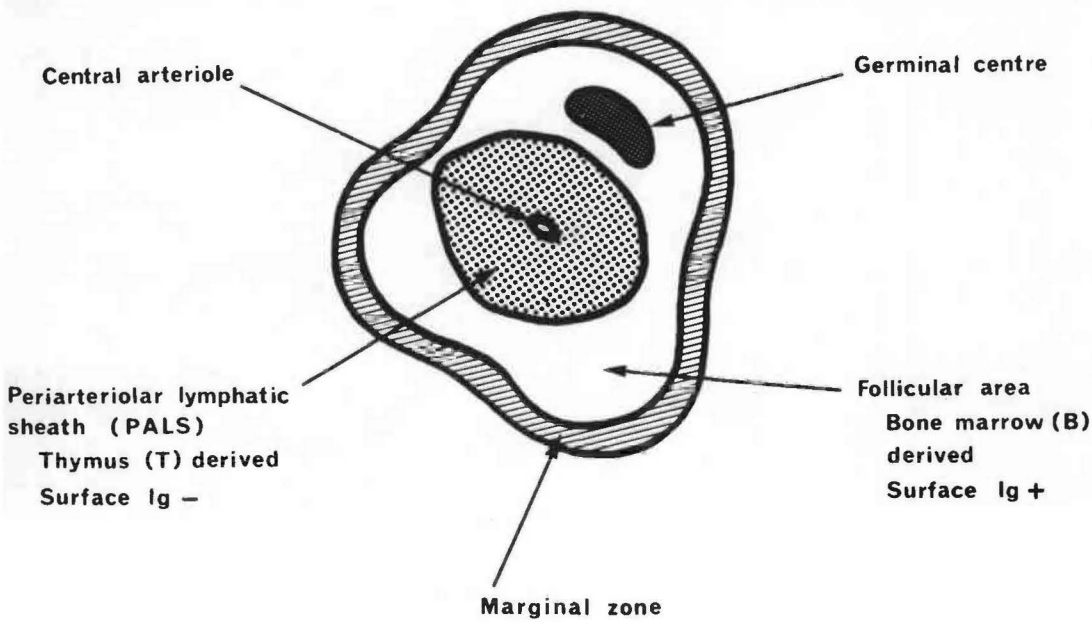

Fig. 2. White pulp compartments of the mouse spleen.

lymphocytes. Thereafter, there was a further increase in content of erythroid cells and an increase in numbers of macrophages and granulocytes. Although the percentage of small and medium lymphocytes continued to fall, the absolute numbers of these cells did not decrease.

Based on these findings, further studies were carried out concentrating on the spleens of *T. congolense*-infected C3H/He mice. Tissues were examined by standard histologic techniques and by immunofluorescence for cytoplasmic and cell surface Ig using the methods of Gutman and Weissman (1972). Our findings showed that the splenic cellular changes could be divided into two distinct phases: an early proliferative phase involving both the white and the red pulp and a later phase characterized by a gradual cellular depletion of the white pulp.

Proliferative Phase

Profound alterations occur in both the white and red pulp of the spleen during the first 10 days of infection. By day 4 after infection, small foci of large blast cells are found in the white pulp, both in the thymus-dependent periarteriolar lymphatic sheath (PALS) and in the B-dependent follicular regions. By day 7 the foci have expanded and in the PALS large pyroninophilic cells can be seen. By immunofluorescence numerous Ig-containing cells are found within the PALS at this time. The changes that occur between day 7 and day 9 are most dramatic. There is a marked proliferative response particularly in the follicular areas, which by day 9 are composed almost entirely of large lymphoid cells showing a high rate of mitosis. Large numbers of dead and dying cells are found throughout the follicular areas, and there are numerous tingible body-containing macrophages. By immunofluorescence, there is a marked increase in the number of Ig-containing cells found in the PALS (Fig. 4) and in the red pulp; however, the follicular areas that are strongly positive for cell surface Ig in normal mice and in infected mice up until day 7 show a marked loss in intensity of staining for surface Ig by day 9 (Fig. 5). By day 11 only small numbers of cells showing low intensity surface Ig staining are found in the follicular areas. Throughout this period well circumscribed germinal centres cannot be found and by immunofluores-

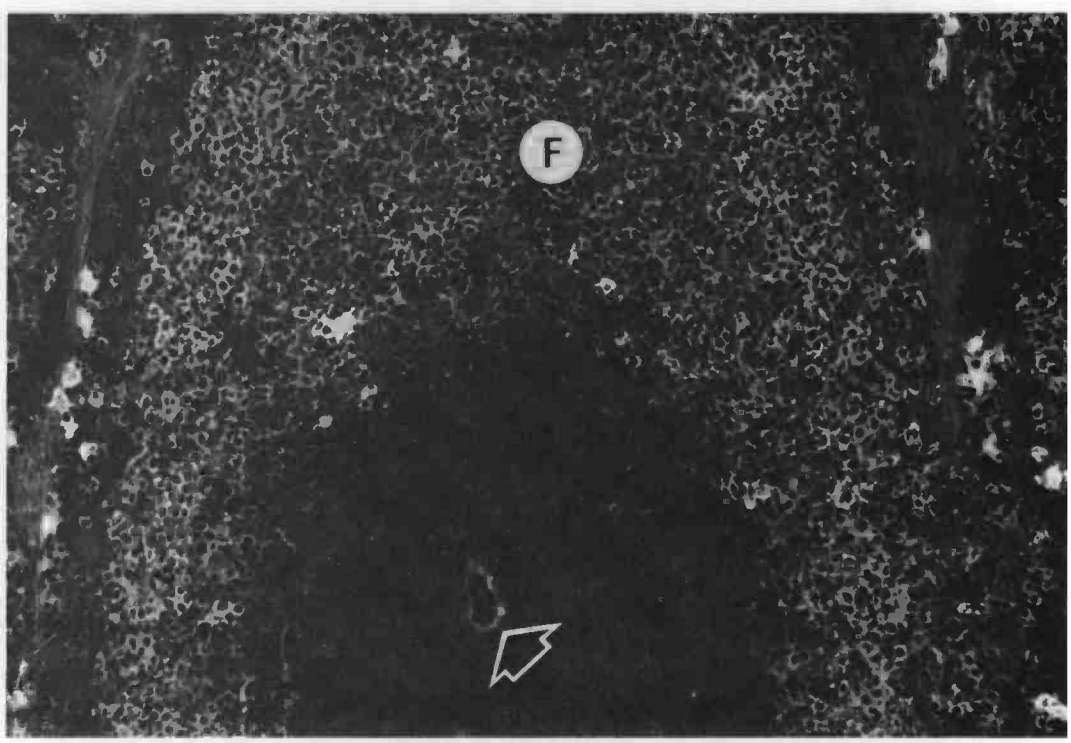

Fig. 3. *Section of normal mouse spleen stained by immunofluorescence for mouse Ig. Cells in the PALS, surrounding the central arteriole (arrow), are negative for surface Ig; those in the follicular area (F) are positive.*

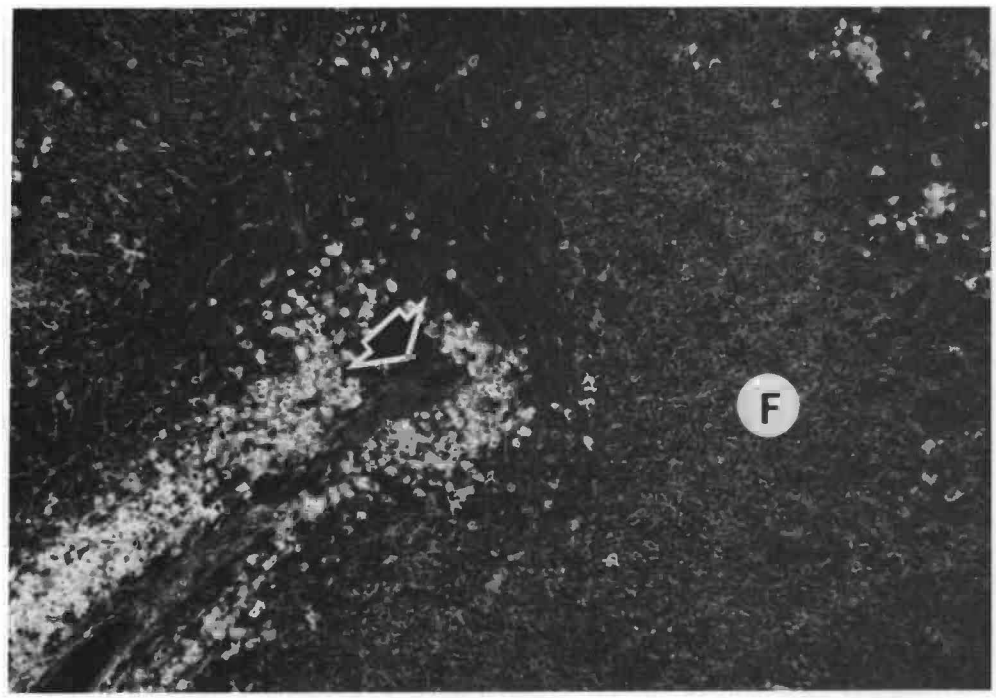

Fig. 4. *Section of mouse spleen 7 days after infection with* T. congolense. *Large numbers of Ig-containing cells are present in the PALS, surrounding the central arteriole (arrow). Follicular area is represented by F.*

cence only small numbers of follicles contain weakly staining Ig deposits on dendritic reticular cells.

During this initial 10 day period there is also a marked expansion of the splenic red pulp that contains large numbers of erythroid precursors and increased numbers of plasma cells.

Depletion Phase

Already by day 11 after infection, the PALS of the white pulp shows some depletion of small lymphocytes. Thereafter, this depletion rapidly progresses so that as early as day 16 this area contains predominantly plasma cells and lymphoblasts with very few small lymphocytes. There is also a gradual but much slower depletion of the follicular areas. Thus, although throughout the infection the follicles contain predominantly large lymphoid cells showing high mitotic activity, they become progressively reduced in size so that in animals with long-standing infection they are exceedingly small and much less cellular. Following the first wave of parasitemia, the follicular cells are largely negative for surface Ig positive cells and only a few follicles contain weak granular deposits of Ig on dendritic reticular cells.

Throughout this period there is further expansion of the red pulp, with a marked increase in red pulp to white pulp ratio. Much of this expansion is due to an increase in erythropoietic cells that, by day 35 of infection, may make up as much as 50% of the total spleen cells. There is also a lesser increase in granulopoietic activity and a gradual increase in the numbers of macrophages so that in cases of advanced infection large numbers of macrophages showing vacuolated cytoplasm can often be found within distended red pulp sinuses. During this period the numbers of plasma cells found in the red pulp gradually decrease.

Functional Implications

Many of the changes described above provide supportive evidence that the functional defects found in in vitro assays are of importance in vivo. However, in many instances they also pose further questions:

Early blastogenic response: On the basis of hypergammaglobulinemia, cellular hyperplasia, and increased background plaque forming cells to sheep RBCs in the spleens of trypanosome-infected mice, it has been suggested that trypanosome infection

induces a polyclonal type of activation involving primarily B cells (Urquhart et al. 1973; Greenwood 1974; Murray, P.K. et al. 1974b; Hudson et al. 1976). This is supported by our finding of a rapid widespread proliferative response involving the entire follicular areas of the white pulp. The marked decrease in intensity of staining for surface Ig on the follicular cells is further indication of a transformation to blast cells. It is unlikely that such an early widespread blastogenesis in this region could be accounted for entirely by specific responses to trypanosome antigens.

Production of large numbers of Ig-containing cells: Following a single antigenic challenge Ig-containing cells can be found within the PALS during the first 4–5 days. Subsequently, most of the Ig-containing cells are found in the red pulp (Van Ewijk et al. 1977). In the present study large numbers of Ig-containing cells were found in the PALS during most of the infection. It is likely that this reflects the continual high level of antigenic challenge encountered by trypanosome-infected animals. However, the massive number of Ig-containing cells found in the PALS at the time of intense proliferative activity in the follicular areas suggests that they may accumulate in this site partly as a consequence of the polyclonal activation. Because we have found that most of these cells contain IgM, it may be that there is also a failure or delay in the switch from IgM to IgG production.

Poor germinal centre formation: At no stage during the infection was the germinal centre activity appropriate to the degree of antigenic challenge, as judged by localization of Ig on dendritic reticular cells. Recent studies indicate that germinal centres may be important in regulating the immune response. Firstly, it has been shown that the localization of antigen–antibody complexes on the dendritic reticular cells in germinal centres is an important step in the generation of memory cells (Klaus and Humphrey 1977; Klaus 1978b). Secondly, on the basis of the finding that antigen–antibody complexes are much more effective than antibody alone in generating anti-idiotype antibody, Klaus (1978a) has suggested that germinal centres are important in initiating anti-idiotype feedback mechanisms. In view of the apparent failure of germinal centre formation in *T. congolense*-infected mice both of these observations could have extremely important implications. Whether the failure to localize immune complexes

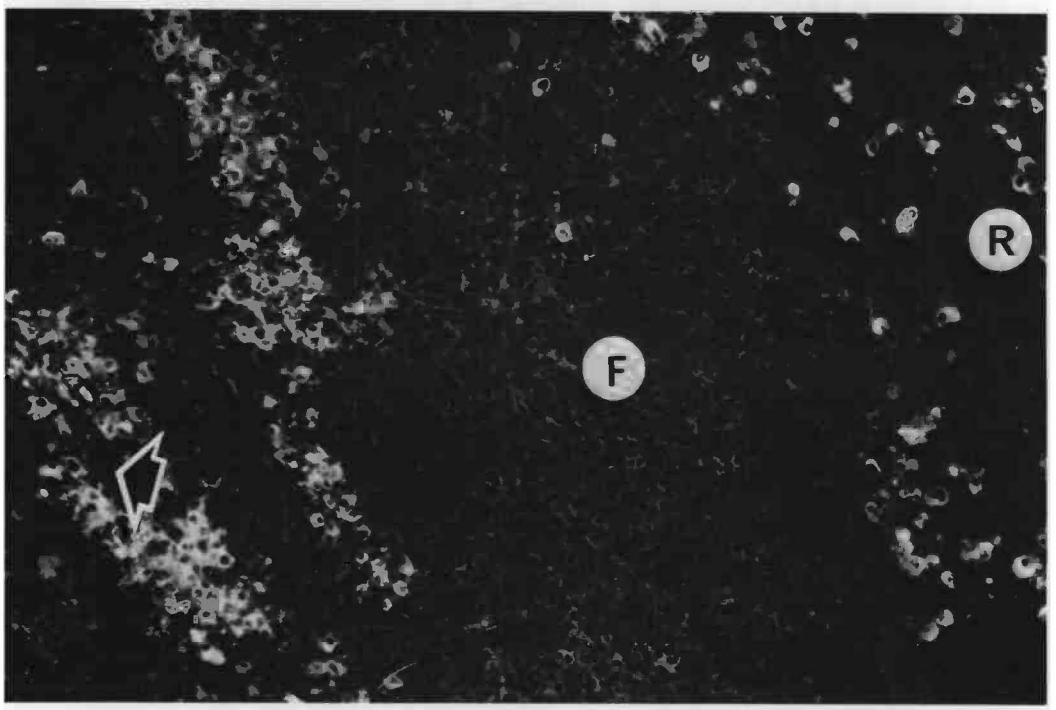

Fig. 5. *Section of mouse spleen 9 days after infection with* T. congolense. *There is marked decrease in intensity of staining for surface Ig in the cells of the follicular area (F). Ig-containing cells can be seen surrounding the central arteriole (arrow). Red pulp is represented by R.*

in germinal centres in infected mice is expressed as a failure to generate memory cells remains to be determined. However, it is of interest that the localization of complexes in germinal centres is complement-dependent (Klaus and Humphrey 1977) and that trypanosome infection results in marked hypocomplementemia (Galvao-Castro, Hochmann, and Lambert 1978). Whatever its cause, this inability to form germinal centres is rapidly reversible; we have found that following treatment of infected mice with Berenil there is rapid repopulation of the follicular areas of the spleen and widespread active germinal centre formation (Roelants et al. submitted for publication).

Depletion of the white pulp: Relatively early in the infection, we found a severe depletion of small lymphocytes from the thymus-dependent PALS. However, at this time, normal or increased numbers of θ-positive cells are found in spleen cell suspensions prepared from infected mice (Roelants et al. 1978). This suggests that there is a different distribution of θ-positive cells in the spleen of infected mice, although at present the localization of these cells is not known. However, it is possible that the θ-positive cells in infected spleens represent an expanded subpopulation of thymus-derived cells but that there is a true depletion of other thymus-derived subpopulations. It has been suggested that the PALS is an important site of cooperation between T and B cells in the immune response (Van Ewijk et al. 1977). If this is so, then the absence of the appropriate T cells from this site could have an adverse effect on the ability of infected mice to respond to bloodborne antigens including the trypanosome itself. In long-standing infections, there is also depletion of the B-dependent follicular areas. Although the depression of various immune responses observed in infected mice does not appear to be due to a depletion of responsive cells (Pearson et al. 1978b) it may be that in advanced infections this is not entirely the case. This apparent contradiction needs to be resolved. Treatment of infected mice with Berenil results in a rapid repopulation of the white pulp, particularly the follicular areas, by lymphocytes (Roelants et al. submitted for publication). This is associated with a return to normal immune responsiveness. It is not known whether the precursors of these repopulating cells are present in the spleen before treatment or whether there is recruitment of cells from the circulating pool. In this respect, it would be interesting to know whether changes occur in the circulating lymphocyte population and whether or not there is any defect in recruitment of lymphocytes into the spleens of infected mice. Furthermore, there is no available information on the output of newly produced lymphocytes from the bone marrow in infected mice.

Erythropoietic response: The extensive erythropoietic activity observed in the spleens of infected mice undoubtedly represents a response to anemia resulting from increased red blood cell breakdown. Indeed, macrophages engaged in erythrophagocytosis are often observed alongside the erythroid precursors in the red pulp of the spleen. However, whether or not the presence of such large number of erythropoietic cells has any effect on the migration of lymphoid cells within the spleen or on the potential proliferation of lymphoid cells upon antigenic stimulation is at present unknown.

Changes in the Lymph Nodes

In studies of the immune response in animals infected with trypanosomes, the results obtained with spleen cells are often considered as being representative of the overall status of the immune system. However, at least in *T. congolense* infection of mice, this is not the case. Indeed, the lymph nodes of *T. congolense*-infected mice show only minor changes before day 20 of infection. However, from about day 20 onward, marked proliferative activity starts in the follicular cortex and numerous plasma cells appear in the medullary cords. As in the spleen, the follicles contain predominantly blast cells. At this time, the thymus-dependent paracortical areas are still populated predominantly by small lymphocytes and show only focal proliferative activity. These changes become more pronounced as the infection progresses; in long-standing infections, plasma cells are found in large numbers not only within distended medullary cords but also in the inner paracortical areas. Although there is some narrowing of the cortex and a reduction in size of follicles, the degree of depletion is not so severe as that observed in the spleen.

Because *T. congolense* is thought to be confined entirely to the circulation, proliferative activity in response to the trypanosome may occur either as a result of stimulation by soluble antigens entering the nodes from the circulation or by migration into the nodes of antigen-primed cells from the circulation. In either case it is difficult to explain why there is a strong proliferative response in the lymph nodes only after 2–3 weeks of infection. It would be of interest to learn whether lymph node cells undergo the same functional changes as are observed in the spleen. Preliminary results indicate that depression of immune responsiveness occurs

much later in the lymph nodes, but whether the same mechanisms are operative as in the spleen remains to be determined. Furthermore it is not yet known whether, as in the spleen, there is a defect in trapping of immune complexes by the follicular dendritic reticular cells and whether the Ig response is also predominantly of the IgM class.

It should be remembered that, in *T. brucei* infections, during which the organisms enter the tissues and are present in lymph (Lambert and Houba 1974; Ssenyonga and Adam 1975), the changes observed in the lymph nodes are much more severe and occur much earlier than with *T. congolense* (Murray, M. et al. 1974; Murray and Morrison unpublished data). A detailed comparative study of the lymph nodes in these two infections might, therefore, be of value.

Changes in the Lymphoid Organs of Cattle

At present, much less is known about the effect of trypanosome infections on the immune system of cattle than in laboratory animals. However, there are reports in the literature of decreased immune responsiveness in trypanosome-infected cattle (Holmes et al. 1974; Scott et al. 1978) although the magnitude of the immune depression appears to be much less than that observed in laboratory animals.

During the first 3–4 months following experimental infection of cattle with either *T. congolense* or *T. brucei* there is marked proliferative activity in the lymphoid organs. The follicular areas show active proliferation with germinal centre formation and numerous plasma cells are found in the medullary cords of the lymph nodes and in the splenic red pulp. However, the proliferative response is much more orderly than that observed in mice. A mantle layer of small lymphocytes remains around the germinal centres and the paracortical areas of the lymph nodes and the periarteriolar areas in the spleen still contain numerous small lymphocytes. In addition, immunofluorescence shows heavy deposits of immunoglobulin within the germinal centres. Thus, at least during the first 3–4 months of infection in cattle there is no morphologic evidence of dysfunction of the immune system. However, on examination of long-standing bovine field cases, in some animals we have found evidence of depletion of the lymphoid organs. The lymph nodes of these animals are small, fibrotic, and relatively acellular; there is a marked reduction in the paracortical areas and the lymphocytic follicles are small and inactive.

Obviously, more extensive studies are required to evaluate adequately the immune system of trypanosome-infected cattle. However, our preliminary observations indicate that functional abnormalities, if they occur, may be much less severe and may take longer to develop in cattle than in laboratory animals. In this respect, any study of the immune system in cattle infected with trypanosomes should be undertaken on a long-term sequential basis.

Changes in the immune system during experimental African trypanosomiasis

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Abstract. Animals infected with African trypanosomes are often severely immunodepressed. In this paper we summarize some of our own studies on this immunodepression, discuss several of the hypotheses used to explain it, and speculate on its nature and experiments that would lead to a better understanding of it.

All the trypanosome species and isolates cause changes in the cells and structure of the lymphoid system, in the levels of serum immunoglobulin, and in functional immunity, although the degree varies from one host species to another. In trypanosome-infected laboratory animals, virtually every part of the immune system shows functional abnormalities. In this paper, we present our data on trypanosome-induced immunodepression in mice infected with *T. congolense* and then attempt to relate the data from our lab and others to possible mechanisms involved. We also speculate on experiments that should be done.

To What Extent are Trypanosome-Infected Mice Immunosuppressed?

Many studies have shown that B-lymphocyte responses to antigens are decreased in trypanosome-infected animals (Goodwin 1970; Urquhart et al. 1973). Studies with the B-lymphocyte mitogen lipopolysaccharide (LPS) indicate that the B cell compartment is hyporeactive (Murray P.K. et al. 1974b; Albright, Albright, and Dusanic 1977; Jayawardena and Waksman 1977). A few limited studies with T cell mitogens indicate that T-lymphocyte responses are similarly depressed in cells from infected animals. We examined T-lymphocyte responses more closely than previous studies. We used C3H/Tif or CBA mice infected with *T. congolense* (Morrison et al. 1978). The isolate of *T. congolense* that we chose produced in the mice long-term infections that resemble *T. congolense* infections in cattle, which are usually chronic. At intervals after infection, we measured DNA synthesis in spleen cell suspensions that were cultured in vitro with the mitogen Concanavalin A (ConA), with allogeneic spleen cells differing at the H-2 locus, and with cells identical at the H-2 locus but differing at the minor lymphocyte-stimulating locus (Mls). We also measured cytotoxic lymphocyte activity after in vitro culture of spleen cells with normal allogeneic (H-2 different) spleen cells as either stimulators or responders. To test T-lymphocyte function in vivo, we performed allogeneic skin grafts in infected mice. All tests measured T-lymphocyte function. The results published elsewhere (Pearson et al. 1978) are summarized here:

- Stimulation of T-lymphocytes by ConA or in MLR was severely depressed or abolished in infected mice as early as 9 days after infection.
- Cytotoxic lymphocytes were not generated in MLR when spleen cells from infected mice were used as responder cells.
- Mean allograft survival time and rejection period were both prolonged in infected mice.
- Complete mitogen dose and time curve analysis showed that the decreased responses were not simply due to a shift in mitogen concentration or in time required for triggering DNA synthesis.

Analysis in mixed lymphocyte culture showed that spleen cells from infected mice failed to *stimulate*. Immunofluorescence studies indicated

that the failure to respond or to stimulate was not due to reduced numbers of the relevant T- or B-lymphocytes (Roelants et al. in press). We found strong suppressor cell activity in spleens of *T. congolense*-infected animals (Pearson et al. in press). The suppression was generalized (suppressed both B- and T-lymphocyte responses to mitogens and T-lymphocyte responses in MLR) and was not H-2 restricted. Mice infected with *T. congolense* showed depressed responsiveness to all mitogens and antigens tested so far in vitro and to allogeneic skin grafts in vivo.

What causes the Immunodepression in Trypanosome-Infected Mice?

A number of hypotheses have been put forward to explain immunodepression. For instance, the tremendous polyclonal activation of B-lymphocytes (Hudson et al. 1976) in infected animals has led some authors to suggest that "exhaustion" of B-lymphocyte potential occurs, resulting in lack of response to B cell mitogens and to antigens (Corsini et al. 1977). However, "clonal exhaustion" is not absolute, as B cell responses to some helper T cell-independent antigens do occur in some systems (Mansfield and Bagasra 1978). T-lymphocytes also increase in spleens of trypanosome-infected mice (Morrison et al. 1978), and, presumably "clonal exhaustion" of T-lymphocyte potential also occurs. Again it does not appear to be absolute as low responses in MLR are obtained even in animals with long-standing infection (Pearson et al. 1978).

Our recent experiments show that spleen cells from infected mice rapidly regain immune reactivity after treatment with the trypanocidal drug Berenil (Roelants et al. submitted for publication). The return to normal activity would probably not be so rapid if "clonal exhaustion" was the sole mechanism causing hyporeactivity.

In spleens of infected mice, there is a moderate increase in the numbers of T-lymphocytes and macrophages, a considerable increase in B-lymphocytes, and a tremendous increase in "null" cells (Morrison et al. 1978). The B- and T-lymphocytes may be diluted and, thus, not present in large enough numbers to respond to the various mitogens or antigens tested in vitro.

We found by immunofluorescence that there was no shortage of T-lymphocytes in spleens of infected mice (Pearson et al. 1978; Roelants et al. in press), but there may have been selective loss of responding T-lymphocytes and replacement with other Thy. 1 bearing cells.

The B-lymphocytes in infected mouse spleens were sufficient to induce stimulation in MLR; they did not do so, however, unless they were purified by agglutination with soybean agglutinin (Roelants et al. in press). These experiments indicate that dilution of the relevant cells is unlikely to be the cause of immunodepression and led us to look for other mechanisms.

Active suppression of normal spleen cell responses has been well documented in mice infected with *T. brucei* (Jayawardena and Waksman 1977; Corsini et al. 1977) and with *T. congolense* (Pearson et al. in press). With *T. congolense*, the suppression is generalized, not antigen specific and not H-2 restricted. The nature of the suppressing cells remains unresolved but they may be macrophages or T cells or both (Pearson et al. in press).

Mitogenesis has been induced in spleen cells in vitro by extracts of *T. brucei* (Esuruoso 1976) and by autolysates of *T. congolense* (Assoku, Tizard, and Nielsen 1977; Tizard et al. p. 103). That this stimulation is responsible for reduced immune responsiveness by causing "immune exhaustion" is possible as are other direct or indirect mechanisms, although the relevance of mitogenesis in vitro to the in vivo situation has not been determined. Albright, Albright, and Dusanic (1978) suggest that soluble substances derived from *T. musculi* act directly on B-lymphocytes or "helper" cells and not indirectly through suppressor T cells or macrophages. They found that suppression was induced by cell populations containing significant numbers of parasites. In *T. congolense* infections, we found few parasites in the spleens, and we observed that lymph node cells remain immunoreactive long after spleen cells are depressed. These two observations lead us to believe that the mechanism in *T. congolense* infections is not simply a direct suppression by soluble factors. In any case, indirect mechanisms of suppression by soluble "factors" should be investigated.

Some or all the mechanisms discussed may account for the depressed immune responsiveness in trypanosome infections; it is also possible that none of them is the primary mechanism. One thing is clear: no single explanation is without contradiction in the available literature.

Some recent experiments in our lab lend support to the idea that immune responsiveness (in this case suppression) is actively regulated in *T. congolense*-infected mice. The results will be published elsewhere (Roelants et al. submitted for publication) but, in brief, are:

- Depressed responses to B and T cell mitogens and in MLR parallel the appearance of trypano-

somes in peripheral blood.

- Suppressor cell activity parallels the appearance of trypanosomes in peripheral blood.
- Treatment of infected mice with the trypanocide Berenil eliminates trypanosomes, restores immune reactivity, and abolishes suppressor cell activity within days. A return to normal lymphoid architecture also occurs.

The rate at which normal immune reactivity is restored in treated mice suggests a regulatory mechanism because the elimination or inactivation of responding cells would require that a longer period is needed for restoration to a functional state.

Is the Immune System of Infected Mice Functioning as It Was Designed?

Perhaps trypanosome-infected mice respond to the onslaught of parasites by normal immune reactivity. That is, the observed immune depression in infected animals may be a consequence of suppression or "down regulation" of the massive polyclonal activation induced during infection. There are several facts that point to a "normal" response. The immune depression and suppression is generalized, not antigen specific and not H-2 restricted. Cell-mediated responses are also depressed and, because cell-mediated immunity to trypanosomes has not been demonstrated, depression of these responses seems to be unnecessary for the survival of the trypanosome. Immune complexes are often found in infected animals, and antigen–antibody complexes are thought to be ideal for induction of anti-idiotype antibody (Klaus 1978a). Autoantibody production occurs in trypanosome-infected mice (Mackenzie and Boreham 1974c).

Idiotype–anti-idiotype regulation mediated through specific suppressor cells or antibodies may

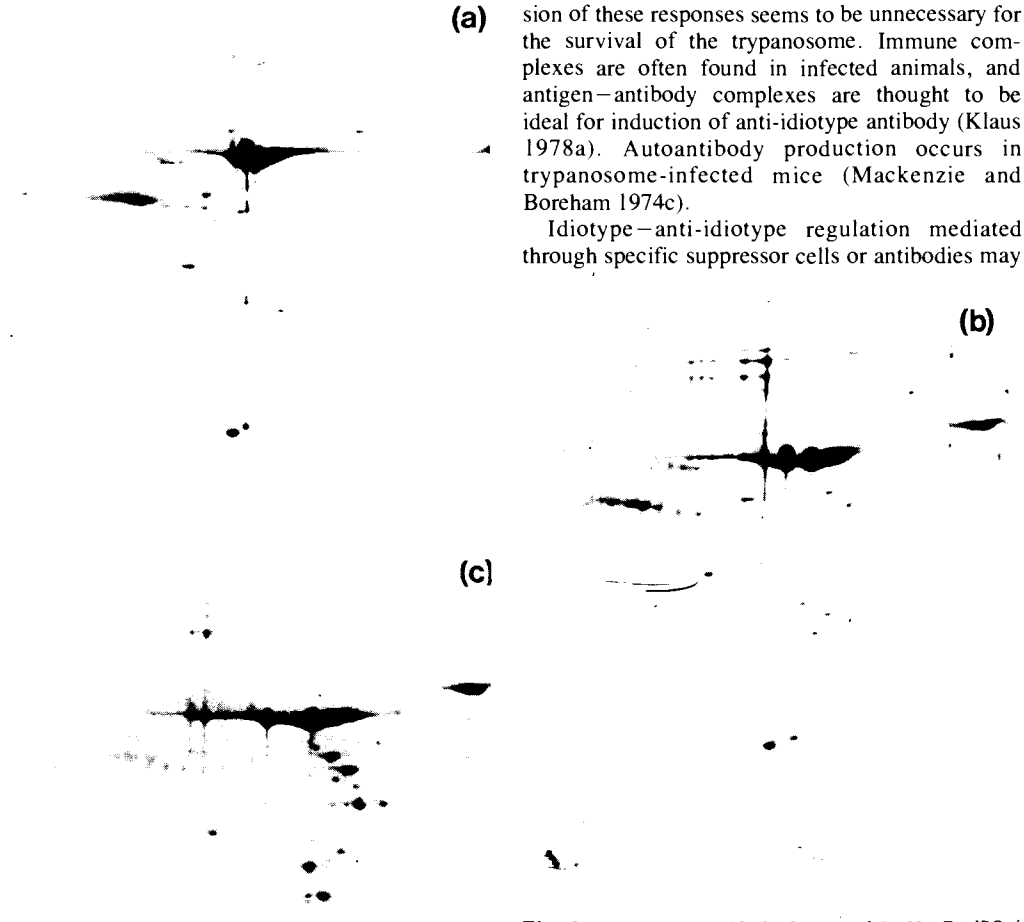

Fig. 1. Fresh serum (10μl) denatured in Na DodSO$_4$/ mercaptoethanol (a) before infection, (b) 7 days after infection with T. congolense, *and (c) 22 days after infection. First dimension (isoelectric focusing): acid end of gel on left; second dimension (SDS-PAGE gel electrophoresis): decreasing molecular weight from top to bottom (technique from Anderson and Anderson 1978a, b).*

function in infected mice to limit the obviously harmful polyclonal proliferation of lymphocytes and production of autoantibody. The parasite may thus "pull the plug out of the bathtub" and set in motion a complex series of events leading to immune depression that may, on balance, be of benefit to the animal host. The depression is beneficial only temporarily, as the host eventually dies due to proliferation of the parasite (murder rather than suicide).

What Should Be Investigated?

All the experiments reported here have sidestepped (perhaps understandably so) the central problem: how does the immune system of infected mice respond to the invading trypanosome? The phenomenon of antigenic variation makes it extremely difficult to measure either antibody or cell-mediated responses to the invading trypanosomes after several parasitemias have occurred. One possible way is to remove aliquots of blood from infected animals at intervals, separate the trypanosomes and serum, and test SDS-lysed and reduced trypanosome preparations and serum on separate, parallel two-dimensional gels. Electrophoresis should spotlight any immunoglobulin (Ig) bound to the trypanosome population. If the host is unable to produce trypanosome-specific antibody as the infection progresses, there should be increasingly less evidence of Ig on the trypanosomes. A simpler approach is to infect (with a trypanosome clone of a known antigenic type) mice that are already infected with antigenically different trypanosomes and then to measure the antibodies to the challenge population. This experiment does not measure the response to the initial infection but shows whether infected animals can respond to any trypanosome antigens.

In infected mice there are many changes in cell populations and evidence that different suppressor cells act at different times (Corsini et al. 1977). A system that allows a kinetic analysis may thus be necessary for detailed understanding of suppressor mechanisms. Isolation of suppressor cells will be facilitated by using monoclonal antibodies to cell subpopulations in concert with the fluorescence-activated cell sorter (FACS).

Detection of immunosuppressive "factors" may be made possible by two-dimensional gel electrophoresis of serum from infected mice. We used 10 ml serum from a single A/J mouse and tested 10μl of it on two-dimensional gels before infecting the mouse and at 7- and 22-day intervals after infection with *T. congolense* (Fig. 1). Several new protein "spots" appeared in the sera of the infected mouse as early as 7 days after infection. The amount of material in these spots increased appreciably by day 22. This system, based on multiple two-dimensional gel electrophoresis (Anderson and Anderson 1978), enables high resolution kinetic analysis of serum changes in infected animals and may lead to isolation of immunoreactive factors.

Finally, we suggest that various lymphoid organs be compared for immune reactivity in infected animals. We have preliminary evidence in several strains of *T. congolense*-infected mice that spleens become hyporeactive long before lymph nodes do. This may explain why infected mice are able to reject allogeneic skin grafts (albeit more slowly than uninfected animals), even though their spleen lymphocytes respond extremely poorly (if at all) in vitro (Pearson et al. 1978). Comparative studies on various lymphoid organs are important because most investigators studying immune responses in humans and cattle use only peripheral blood lymphocytes in their tests, an approach that may be entirely misleading.

Immunosuppression of humoral immune response in bovine trypanosomiasis

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Abstract. The literature on the immunosuppressive effects of *T. congolense* and *T. vivax* infections in cattle is reviewed. The significance to vaccination programs is discussed with reference to diminazene aceturate (Berenil) treatment and immunization with rinderpest vaccine, *Brucella abortus* S19, and *Leptospira biflexa*.

The immunosuppressive effect of African trypanosomiasis is now well documented in laboratory animals; its significance in domestic animals has not yet been determined. The immunosuppression may mean that trypanosomiasis, long recognized as a cause of considerable economic loss in African livestock, is even more lethal and is responsible for even greater losses than previously thought. It may predispose animals to other infections and interfere with their response to vaccinations.

Immunosuppression in Cattle

The earliest evidence of the immunosuppressive effect of trypanosomiasis in cattle was by Parkin and Hornby (1930) who reported that *T. congolense*-infected animals became incapable of thriving and lost condition and resistance to intercurrent infections. This finding was reiterated by Fiennes (1954) who reported bronchopneumonia in cattle suffering from trypanosomiasis. Since then, anaplasmosis, babesiosis, and salmonellosis have also been reported as complications of trypanosomiasis caused by *T. vivax* and *T. congolense* in cattle (Krampitz 1970; Losos 1975).

Direct evidence of the immunosuppressive effect of trypanosomiasis in cattle was first reported by Holmes et al. (1974) who found that the secondary humoral response to polyvalent clostridial vaccine in *T. congolense*-infected cattle was lower than in uninfected controls. They pointed out that this phenomenon may endanger the immunization programs in trypanosomiasis-endemic areas. Subsequent studies using bacterial and viral antigens (Scott et al. 1977; Rurangirwa et al. in press; Whitelaw et al. 1978) have confirmed that cattle suffering from trypanosomiasis have immunosuppressed humoral response to unrelated antigens.

Level of Humoral Unresponsiveness

In laboratory rodents, complete unresponsiveness has been observed after about 3 weeks of infection (Murray P.K. et al. 1974b; Freeman, Hudson, and Byner 1974; Corsini et al. 1977; Mansfield, Craig, and Stelzer 1976; Hudson et al. 1976; Mansfield and Bagasra 1977) and has been attributed either to the polyclonal exhaustion of the B cells by a mitogenic factor of trypanosome origin (Esuruoso 1975; Assoku and Tizard 1978) or to depressed suppressor cells and/or suppressed helper T cells (Mansfield and Bagasra 1977; Jayawardena and Waksman 1977). The cattle that we infected with *T. congolense* and immunized 25 days later mounted a depressed humoral response, which did not fall below 50% of the uninfected controls. The response in *T. vivax*-infected animals was depressed by only 20%. The route of immunization (subcutaneous or intravenous) did not seem to

influence the results, although only one route was used for each antigen. Some work needs to be done using the same antigen for the two routes. We have also found that cattle immunized 15 days after infection, treated with Berenil (7 mg/kg body weight) 10 days later, and then reimmunized 18 days after primary immunization mount anamnestic response, possibly suggesting establishment of "memory" during the infection.

Immunosuppression and Vaccination

Scott et al. (1977) carried out a field vaccination trial in infected cattle using foot-and-mouth and clostridial vaccines. They found that the levels of antibody produced were equivalent to those providing more than 95% protection. Thus, they concluded that despite trypanosome-induced immunosuppression the cattle were likely to derive satisfactory protection from vaccination against foot-and-mouth disease and clostridial infection. In our studies, we found that cattle vaccinated with rinderpest virus vaccine 8 days after infection with *T. congolense* or *T. vivax* had the same virus-neutralizing antibody titres as uninfected controls. Similar results were obtained when cattle were vaccinated with the rinderpest virus vaccine 25 days after infection with *T. vivax*. In contrast, there was a 50% depression of the humoral response to *Brucella abortus* S19 in the *T. congolense*-infected cattle and a 20% depression in the *T. vivax*-infected animals when vaccination was carried out 25 days after infection. The same results were obtained when *Leptospira biflexa* was used as the immunogen.

Hypocomplementemia and Immunosuppression

In Pepys' (1976) review of the role of complement in antibody production in vivo, and in an earlier study (1974) he suggested that decomplementation by cobra venom factor led to suppressed immune response, especially the production of IgG. Papamichail et al. (1976) demonstrated that decomplementation also inhibited the localization of intravenously injected antigens in the follicles of the spleen and thus led to disorganization of the lymphoid tissue and antigen processing.

Circumstantial evidence of low complement levels in trypanosomiasis is found in the work of Rodent and Vallet (1908) who demonstrated that the blood of dogs suffering from nagana was less bactericidal than blood from normal animals. In vivo, complement consumption leading to hypocomplementemia has been reported in trypanosome infections of humans (Greenwood and Whittle 1976b), monkeys (Nagle, Ward, and Linsley 1974), cattle (Kobayashi and Tizard 1976; Tabel, Losos, and Maxie 1977; Nielsen, Sheppard, Tizard, et al. 1978b), mice (Woodruff et al. 1973), and rats (Jarvinen and Dalmasso 1976). In vitro, trypanosomes have activated C2 and C4 (Nielsen, Sheppard, Tizard et al. 1978b; Musoke and Barbet 1977), although their role in complement activation in vivo has not yet been determined.

We have found that, at the first wave of parasitemia, total hemolytic complement and C3 respectively drop to 5.25% and 50% of preinfection levels. They remain low until treatment is instituted at which time they rise rapidly. The role played by hypocomplementemia in the immunosuppression observed in cattle trypanosomiasis is not yet established.

Mechanisms of Immunosuppression

All theories that have been advanced to explain the phenomenon of immunosuppression in African trypanosomiasis are based on studies in laboratory animals. A few are very popular and deserve mention. For instance, Mansfield and Wallace (1974), Mansfield and Bagasra (1977), and Terry (1976) have proposed that unresponsiveness occurs primarily within subpopulations of T-lymphocytes and leads to loss or suppression of helper T cell function. The authors hypothesize that proliferating B cell clones fail to switch from IgM to IgG synthesis and that the suppressor T cell function is lost or depressed. In contrast is the theory of nonspecific B-lymphocyte mitogen associated with trypanosomes, put forward by Urquhart et al. (1973) and supported by others (Greenwood and Whittle 1976b; Corsini et al. 1977). The proponents of this theory hypothesize that a trypanosomal mitogen is responsible for the unregulated proliferation and IgM synthesis of B cell clones and that clonal exhaustion eventually results in the observed suppression of B cell functions. In support of this concept are the reports of Esuruoso (1975) and Assoku and Tizard (1978). These works have demonstrated that *T. brucei* and *T. congolense* extracts are mitogenic in vitro for normal mouse spleen cells and that the mitogenic effects are directed against B cells.

Another theory presently gaining popularity is that trypanosomiasis generates suppressor cells. This was first suggested by Jayawardena and Waksman (1977) and supported by Corsini et al. (1977). Eardley and Jayawardena (1977) have also

demonstrated the presence of suppressor macrophage cells as well as suppressor T cells in the spleens of *T. brucei*-infected mice. They suggest that nonspecific suppressor T cells are stimulated directly by trypanosomes to produce suppressive factors that act through macrophages.

Trypanocidal Chemotherapy and Immunosuppression

Freeman, Hudson, and Byner (1974) demonstrated a complete recovery of in vivo immunocompetence in infected mice that were treated with Berenil and simultaneously immunized with sheep red cells. P.K. Murray et al. (1974) and Ackerman and Seed (1976b) reported recovery within 3 days of trypanocidal chemotherapy. We have found that infected cattle treated with Berenil and then immunized 2 days later mounted a normal response compared to uninfected but treated control animals. Cattle that were infected with *T. congolense*, treated with Berenil on day 25, and immunized 4 days after treatment responded better than did the controls. Of more interest was the humoral response of *T. vivax*-infected cattle when simultaneously treated and intravenously immunized 25 or 50 days after infection. These animals also mounted a significantly better response than did uninfected Berenil-treated control animals.

Discussion

All the basic work on immunosuppression has been done in small laboratory animals using *T. brucei*; however, the most pathogenic trypanosomes of cattle are *T. congolense* and *T. vivax*. All three species cause different syndromes and probably have different tissue distributions. Thus, what one observes in mice/rats using *T. brucei* may not apply to cattle.

Whereas complete immunosuppression is common in mice/rats, so far no reports have shown that immunosuppression in cattle is that extensive. The maximum depression we have observed on one single day was 60% in cattle immunized 25 days after infection. Whether immunosuppression increases with longer infection is not known, although it seems unlikely on the basis of our studies: *T. vivax*-infected cattle experienced more or less spontaneous recovery of immunocompetence on Berenil treatment 50 days after infection. There is a possibility that immunosuppression in cattle is dependent on live trypanosomes. Because parasitemia decreases with time in cattle and may even disappear, the immune response would be less depressed later in the disease. This possibility needs to be verified experimentally.

We compared two routes of immunization (subcutaneous and intravenous) and found the level of depression similar. Thus, one may conclude that the performance of the spleen was the same as that of the peripheral lymph nodes.

The significance of immunosuppression in relation to vaccination programs in cattle is questionable. We have shown that the neutralizing antibody produced by rinderpest virus vaccines was not depressed and that at most immunosuppression to other antigens was only about 50%. Whether the 50% response can be fully protective remains to be established. Because the immunoglobulin catabolic rate is increased in infected calves (Nielsen, Sheppard, Tizard et al. 1978b), the longevity of induced protection is likely to be affected, and more frequent vaccinations would be required.

The role of hypocomplementemia in bovine African trypanosomiasis is unclear. We found that animals vaccinated with rinderpest virus vaccine when their complement levels had reached a minimum mounted a normal response compared to uninfected controls; however, animals immunized with other antigens during low complement levels had depressed responses. (Thus, the role of low complement levels in immunosuppression remains equivocal.) It is most probable that the low complement levels contribute more to predisposition of infected animals to secondary infections than to actual immunosuppression.

The mechanisms of immunosuppression are still not understood even in mice/rats, which have been studied for quite some time. We and others (Whitelaw et al. 1978) have demonstrated that there is more or less spontaneous recovery of immunocompetence when Berenil treatment and immunization are instituted simultaneously. This finding suggests that immunosuppression is dependent on live trypanosomes because effects of a suppressor cell mechanism or of a trypanosomal B cell mitogen would probably not be reversed so soon after treatment. Albright, Albright, and Dusanic (1978) also suggest that the trypanosomes are responsible for the suppressive effect. They have shown that in vitro suppressive effects of T cells or macrophages from *T. musculi*-infected mice are eliminated by incorporating rabbit serum against trypanosomes in the culture.

T. congolense and *T. vivax* infection in cattle do cause immunosuppression. The mechanisms involved during the first 25 days of infection are either dependent on live trypanosomes or susceptible to Berenil treatment, because it has been demonstrated that Berenil overcomes suppression.

The way the trypanosomes effect immunosuppression remains elusive. It seems there is competition between live trypanosomes and the initiation of antigenic immune response. Perhaps the mechanisms involved in the immune response initiation should be delineated and compared to the trypanosomes' metabolism to determine how immunosuppression is effected and why the elimination of the live parasites reverses the effects.

Discussion summary

L. Karstad and V.E.O. Valli

The body's defenses against infection are confounded by trypanosomiasis. In cattle, their ability to counteract the disease varies with breed, age, and possibly environment, and the severity of trypanosome infections varies even among individuals in a homogeneous group. However, a comparison of mild and severe forms of the disease and its effects on the lymphatic system within a large group of homogeneous animals should allow the identification of features that govern susceptibility and resistance.

An effective immune response depends upon the presence of healthy lymphoid tissue. Low levels of serum protein impair the function of lymph tissues and, therefore, hypoproteinemia may account, in part, for the poor response of some trypanosome-infected cattle. This hypoproteinemia could be caused by the glomerulonephritis that is associated with hypocomplementemia, and which is a constant finding in bovine trypanosomiasis. However proteinuria is inconsistently present in bovine trypanosomiasis and therefore the glomerulonephritis is unlikely to be of primary importance.

The germinal centres in lymphoid tissue, which are responsible for the formation and differentiation of lymphocytes, have been shown to be markedly stimulated by *T. brucei*. Their lack of development and poor cellularity in trypanosome-infected animals (see Morrison and Murray p. 154) may be associated with hypocomplementemia, and the observed cellular changes are compatible with a polyclonal stimulation of B cells.

The lymphatic system does not work in isolation but is related to the other hematopoietic cells. Thus, the increased demand for stem cells of the erythroid and myeloid systems may result in stem cell competition with a reduction in lymphoid proliferation. Lymphoid mitoses are reduced in the lymphatic system throughout trypanosome infections while there is an increase in numbers of macrophages.

The immunosuppression is influenced by the suppressor cells, the activity of macrophages, and the occurrence of mixed infections. Comparative work needs to be undertaken on mixed infections as opposed to single experimental infection to determine the importance of immunosuppression in range animals. Immunosuppression may be governed by the duration of infection, although work on antigenic variation indicates that rabbits can produce antibody to the entire sequence of antigenic variants. Secondary and tertiary responses have been studied in *T. vivax* infections where suppression of the antibody response to sheep red cells has been passively transferred by serum in mice.

Macrophages, which appear to be activated in trypanosomiasis, liberate several products with immunosuppressive capacity, including prostaglandins,

interferon, and certain enzymes. These cells efficiently convert arachidonic acid into prostaglandins and may be the means by which unsaturated fatty acids exert their effects in trypanosomiasis. However, other cell types, including T-lymphocytes, K cells, and NK cells, also exert suppressive effects on immune responses. It seems likely that several of these components operate in parasitic infections with tneir relative importance depending on species of parasite and host, the duration of infection, and other unknown factors. It is essential that we determine whether immunosuppression is important in cattle as well as in other animals with chronic progressive trypanosomiasis.

Pathogenesis of tissue lesions in *T. brucei* infections

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Abstract. *T. brucei* is capable of producing tissue damage in a large number of mammalian hosts including the bovine. However, in our experience, the dog is particularly severely affected. In the dog, *T. brucei* causes an acute disease syndrome characterized by high levels of parasitemia and invasion of a wide range of tissues by the trypanosomes. This is associated with marked cellular infiltration and tissue cell degeneration and death. The heart, choroid plexus, and eyes are consistently and severely affected, and a striking finding in some animals is a necrotizing vasculitis affecting the coronary vessels. The mechanisms involved in tissue injury are open to speculation but possibly include immunologic mechanisms, biologically active factors produced by the trypanosomes, physical swelling and disruption of tissue architecture, and increased vascular permeability. Associated with tissue lesions, dramatic lymph node changes occur: initially these are proliferative but later lymphoid depletion occurs.

Organisms of the *T. brucei* subgroup live both extravascularly and intravascularly in their mammalian hosts. In extravascular sites they cause cellular infiltration and, often, tissue cell degeneration and death. The degree to which they invade extravascular sites varies considerably from one host species to another as does the extent of tissue damage. The number of organisms present in the tissues does not always correlate with the number in the blood. In our experience, working with several laboratory and domestic animal species, the most severe tissue lesions occur in dogs infected with *T. brucei* (Morrison et al. in preparation). The purpose of this paper is to describe the main features of tissue lesions found in *T. brucei* infected animals, with particular reference to the dog, and to discuss the possible mechanisms involved in the tissue injury.

Dogs Infected with *T. brucei*

Using two different isolates of *T. brucei*, we have experimentally produced an acute disease in dogs invariably leading to death 3–4 weeks after infection. Both the clinical and pathological findings closely parallel those found in natural cases of the disease. Infected dogs show high levels of parasitemia, and during the 2nd and 3rd weeks of infection there is invasion of a wide range of tissues by large numbers of organisms. In animals examined post mortem during the 3rd and 4th weeks of infection, trypanosomes can readily be found in fluid taken from the peritoneal, thoracic, and pericardial cavities; synovial fluid; cerebrospinal fluid; aqueous humour; and subcutaneous edema fluid. Large numbers of trypanosomes can be seen histologically in many tissues. However, despite the widespread distribution of the organisms, there appears to be some predilection for certain tissues: we have most consistently found severe lesions in the heart, central nervous system, eyes, skin and subcutis, skeletal muscle, kidney, nasal mucosa, testicles, and pituitary gland. Some of the tissues, in particular the heart, central nervous system, skeletal muscle, and pituitary gland are also severely damaged in other species infected with *T. brucei* (Ikede and Losos 1972, 1975; Murray 1974; Murray, P.K. et al. 1974; Poltera, Owor, and Cox 1977).

In the dog, the heart, central nervous system, and eyes are always severely affected. There is a diffuse myocarditis (Fig. 1), which in most terminal cases is judged to be the cause of death. In the central nervous system, the choroid plexus is particularly involved, although lesions are also

Fig. 1. *Ventricular myocardium of dog examined 23 days after inoculation with* T. brucei. *Marked cellular infiltration can be seen throughout the myocardium resulting in separation of the muscle fibres.*

found elsewhere, mainly in the meninges. In the eyes, there is a dramatic anterior uveitis with exudation of cells and fibrin and sometimes hemorrhage into the anterior chamber.

The tissue lesions vary in severity with the numbers of trypanosomes present but, in general, are similar in different tissues. Initially, the cellular infiltrate is composed predominantly of lymphocytes and plasma cells with occasional macrophages. As extravascular trypanosomes become more numerous, large numbers of macrophages and polymorphonuclear leukocytes are found (Fig. 2). In different sites within the same tissue, either cell type may predominate. In the heart, lesions are initially found beneath the epicardium and endocardium and are more severe in the atria than in the ventricles; in more advanced cases the entire myocardium is involved (Fig. 1); there is marked distortion and degeneration of myocardial fibres; and in some sites frank necrosis is apparent. The lymphatics in the epicardium are distended and contain numerous macrophages and polymorphonuclear leukocytes and sometimes lymphocytes and trypanosomes. On occasion, the lymphatics are completely occluded by thrombi that contain fibrin as well as many of the above cell types. An additional feature in the heart is a severe necrotizing vasculitis affecting both arteries and veins in the epicardium (Fig. 3). Affected vessels often exhibit necrosis of the entire vessel wall, which contains large numbers of polymorphonuclear leukocytes and numerous trypanosomes.

Mechanisms of Tissue Injury

Although much has been written on the lesions that develop in trypanosome-infected animals, there have been few attempts to investigate the mechanisms involved in tissue injury.

Physical damage: In infected dogs, the degree of interstitial edema and cellular infiltration observed in many tissues must affect the normal physiological functioning of the tissues. For example, swelling of the choroid plexus causes the vessels in the plexus to separate from the ependymal lining and must affect its normal specialized secretory function. Similarly, in the eye, severe lesions in the choroid, iris, and ciliary body will affect the fluid balance within the eyeball, and indeed some animals suffer from a transient glaucoma. The

anemia, although moderate, is likely to lead to tissue anoxia and contribute to the cellular degeneration. However, these physical effects hardly account for all of the degenerative changes in the tissues.

Increased vascular permeability: As evidence of increased vascular permeability in trypanosome-infected animals, the blood vessel walls are often swollen, with expanded perivascular spaces. This may be, at least partially, related to increased levels of kinins (Boreham 1968a, 1970; Goodwin 1970) possibly brought about by the interaction of antigen–antibody complexes and Hageman factor (Goodwin 1970). In *T. brucei* infection, the local inflammatory response initiated by extravascular trypanosomes is also likely to be an important mechanism, not only in increasing vascular permeability but also in allowing extravasation of leukocytes. Although a localized increase in vascular permeability is a normal physiological response to an invading organism, it may impair normal functional activity if it is severe and prolonged. Its effect could be particularly relevant to highly specialized vascular areas such as the choroid plexus and the uveal tract of the eye. Also, interstitial edema in the heart may interfere with the conducting system by separating Purkinje's fibres from the myocardial fibres.

Direct toxic damage: Until recently, the hypothesis that trypanosome "toxins" are involved in the pathogenesis of trypanosomiasis has been out of favour, especially as early reports were difficult to confirm. However, there are now several studies that describe the existence of biologically active substances produced by either dead (dying) or living trypanosomes. A hemolytic factor, a heat-stable protein with a molecular weight of 10 000 daltons, has been shown to be produced by *T. brucei* (Huan et al. 1975) as well as by *T. congolense*, *T. vivax*, *T. gambiense*, and *T. rhodesiense* (Murray, Huan, Lambert et al. in press). In addition, permeability and inflammatory factors have been demonstrated in association with *T. gambiense* (Seed 1969) and *T. congolense* (Tizard and Ringleberg 1973; Tizard and Holmes 1977), the inflammatory factor being a polypeptide with molecular weight 1500 daltons. Musoke and Barbet (1977) have shown that purified variable antigen of *T. brucei* is capable of activating complement and inducing increased vascular permeability, and Nielsen and Sheppard (1977) have shown that factors extracted from *T. congolense* also activate complement. Davis et al. (1974) demonstrated a heat-labile, non complement-dependent, platelet aggregating factor produced by *T. rhodesiense*, and Ackerman and Seed (1976) found that tryptophol, a substance known to be

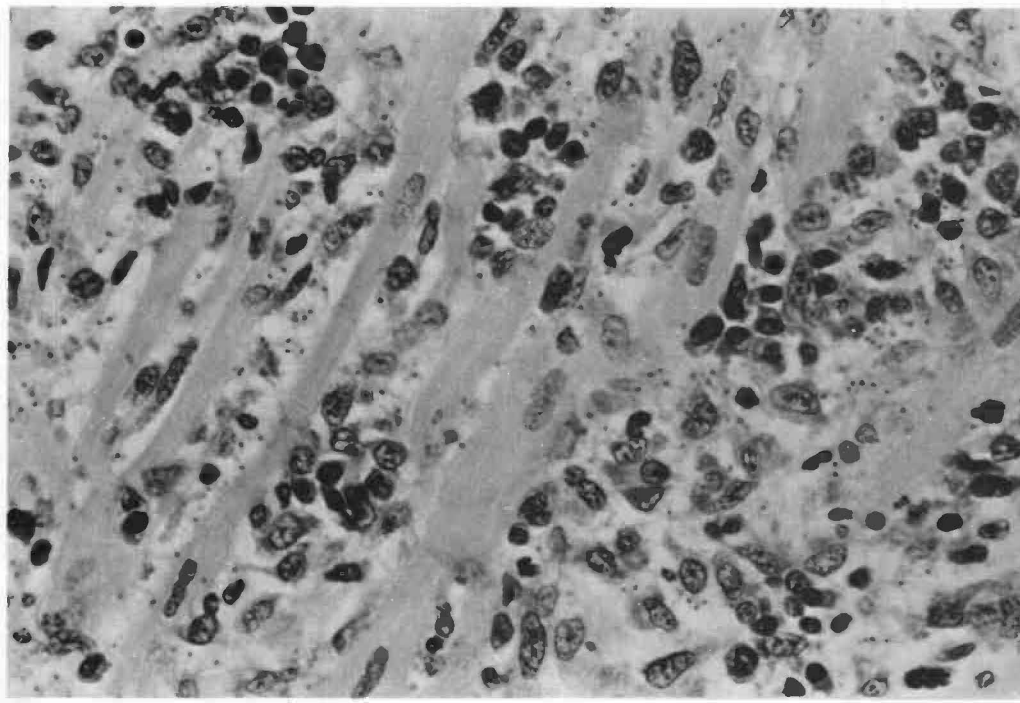

Fig. 2. *Myocardium of dog examined 23 days after inoculation with* T. brucei. *Large numbers of trypanosome nuclei, lymphocytes, plasma cells, and macrophages can be seen between the myocardial fibres.*

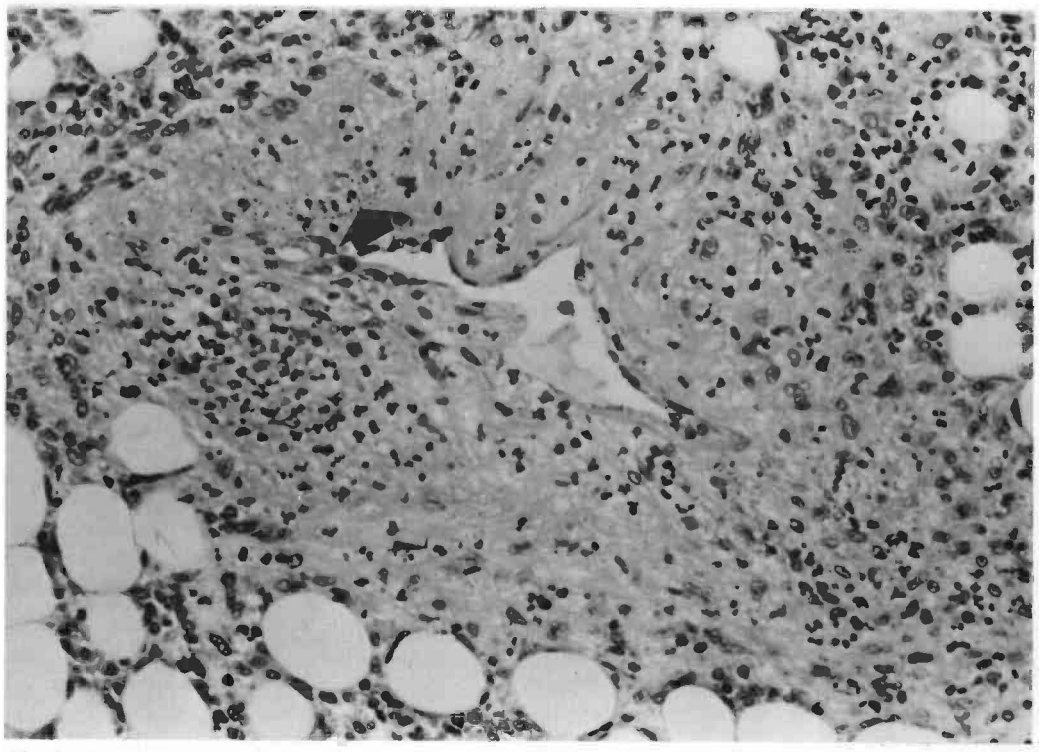

Fig. 3. Artery in epicardium of dog examined 24 days after inoculation with T. brucei. *There is diffuse necrosis of the arterial wall, which contains large numbers of polymorphonuclear leukocytes. Foci of trypanosome nuclei can also be seen (arrow).*

synthesized by *T. gambiense*, induced immunosuppression in laboratory rodents. There is now preliminary evidence that *T. brucei, T. congolense,* and *T. rhodesiense* or their extracts (Esuruoso 1976; Mansfield, Craig, and Stelzer 1976; Campbell and Phillips 1976) act as polyclonal B-lymphocyte mitogens. In a series of papers, Tizard and his colleagues (Tizard and Holmes 1976; Tizard et al. 1978) demonstrated that autolyzing *T. congolense* generate phospholipase A activity and free fatty acids that have hemolytic as well as cytotoxic activity and have been incriminated in the induction of immunosuppression (Assoku, Tizard, and Nielsen 1977). Thus, trypanosomes per se generate factors that may contribute to some of the major lesions known to be involved in the disease process of African trypanosomiasis, including anemia, increased permeability of the peripheral vasculature, complement reactivity, immunosuppression, polyclonal mitogenicity (reviewed by Murray 1974), and thrombocytopenia (Davis et al. 1974), although in many instances their activity in vivo remains to be determined. At present, there is little evidence that trypanosome toxins are involved in the development of tissue lesions. Studies in neonatal mice or sublethally irradiated mice infected with *T. brucei* have demonstrated large numbers of organisms in the tissues but scanty cellular infiltration and virtually no tissue cell degeneration or death (Galvao-Castro, Hochmann, and Lambert 1978). Although these findings are open to interpretation, they suggest that live trypanosomes by themselves are not responsible for severe tissue injury. However, it is possible that when many organisms die in the tissues they release substances capable of initiating tissue damage.

Immunologic mechanisms: The presence of a foreign antigen or organism extravascularly in the tissues initiates a localized inflammatory response that allows serum components and cells to enter the tissues; antigens or antigen-bearing cells leave the tissue by afferent lymphatics and initiate an immunologic response in the drainage lymph node. The resultant antibody or antigen-primed cells may then recirculate from the lymph stream and enter the site of foreign antigen from the bloodstream. In this way, nonspecific inflammatory reactions and specific immune responses complement each other in combatting foreign antigenic challenge. The cells present in the tissues at a particular time

represent a net balance between those entering from the circulation and those leaving in the afferent lymphatics. The balance is dependent on vascular permeability, on stimuli for cells to enter the tissues, and on stimuli for retention of the extravasated cells within the tissues. The type of antigen, the quantity of antigen, and the quality of the host's immune response influence the entire process. In most infectious diseases there is a distinct lack of information on these aspects of tissue reactions in relation to pathogenesis of disease. This may reflect the practical difficulties in carrying out the in vivo studies.

Although in most instances the host's response eliminates a foreign antigen, it is confounded in trypanosome infections by antigenic variation. The immune reaction, which at the outset is beneficial to the host, may actually have adverse effects when it is sustained and intensified.

Support for this possibility comes from recent work carried out in mice infected by *T. brucei* (Galvao-Castro, Hochmann, and Lambert 1978). *T. brucei* produces relatively severe myocardial and skeletal muscle lesions in mice, associated with extravascular trypanosomes and a cellular infiltrate composed of lymphocytes, plasma cells, macrophages, and a few polymorphonuclear leukocytes (Murray 1974; Galvao-Castro, Hochmann, and Lambert 1978). Galvao-Castro, Hochmann, and Lambert (1978) demonstrated granular deposits of immunoglobulin and trypanosome antigen between the muscle fibres in sections of heart and skeletal muscle examined by immunofluorescence. They also found that acid eluates of the tissues contained high levels of antitrypanosome antibodies. In addition, immunologically incompetent mice, including neonates, sublethally irradiated mice, and athymic nude mice, showed lower levels of Ig deposits in the tissues and much less severe tissue lesions despite the presence of large numbers of trypanosomes in the tissues. Furthermore, following the transfer of normal syngeneic spleen cells or an Ig-negative fraction of spleen cells to nude mice, the tissue lesions found, following *T. brucei* infection, were similar to those in intact infected mice. Athymic nude mice that received antitrypanosome antibody 5–7 days after infection showed only slightly more severe lesions than the infected nude controls.

Several mechanisms may be involved: antigen–antibody reactions in the circulation, antigen–antibody reactions within the tissues, cell-mediated reactions, and autoimmune reactions. It is well established that immune complexes are present within the circulation of animals suffering from trypanosomiasis (Galvao-Castro, Hochmann, and Lambert 1978) and that in some instances immune complex deposition occurs in the renal glomeruli (Nagle et al. 1974; Lambert and Houba 1974; Murray 1974; Murray, Lambert, and Morrison 1975). However, such deposits are often detected only after several weeks of infection. In the dogs we examined, only scanty deposits of immunoglobulin were found in the kidneys in a few of the terminal cases. Thus, preformed immune complexes deposited from the circulation are probably not a major mechanism of tissue injury. Much more likely is the local interaction of antibody with trypanosomes or trypanosome antigens within the tissues. This is supported by the observation of Galvao-Castro and colleagues that the severity of the lesions was related quantitatively to the amounts of Ig deposits found in the tissues. The antibody may enter the tissues from the circulation or may be produced locally by plasma cells in the cell infiltrates. Further evidence for local immunologic reactivity was the necrotizing vasculitis that we found in dogs infected with *T. brucei*; this was characteristic of an Arthus reaction. Because large numbers of trypanosomes were found within the vessel walls, it is likely that the vasculitis developed as a result of antibody from the circulation interacting with trypanosome antigen in the vessel walls. We want to emphasize that only in the dog have we seen a true necrotizing lesion, although we have observed a range of vascular degenerative changes in other animals infected with *T. brucei*.

Galvao-Castro and his colleagues' finding that Ig-negative spleen cells increased the severity of lesions in nude mice may have been due to enhanced lymphocyte cooperation that caused greater production of antibody and, hence, larger deposits of immunoglobulin in the tissues. Whether or not conventional cell-mediated reactions are important in the pathogenesis of the tissue lesions remains to be determined. However, a delayed hypersensitivity-type reaction to trypanosome antigen has been demonstrated in rabbits infected with *T. brucei* (Tizard and Soltys 1971).

Various autoantibodies have been detected in the sera of trypanosome-infected rabbits (Mansfield and Kreier 1972; Mackenzie and Boreham 1974c), monkeys and humans (Houba and Allison 1966; Houba, Brown, and Allison 1969; Lindsley, Kysela, and Steinberg 1974), and it has been suggested that they are involved in the development of the tissue lesions. However, the role played by autoantibodies awaits evaluation; it is not known whether they are involved in initiating the tissue lesions or whether they are by-products of tissue antigen being released at sites of injury or, alternatively, are produced during polyclonal B cell activation. In studies of *T. brucei*-infected mice, autoantibodies

to heart and skeletal muscle were not detected (Galvao-Castro, Hochmann, and Lambert 1978). Thus, autoimmune reactions, if they are involved at all, may only be operative in long-standing infections when there are severe changes in the lymphoid system.

In dogs infected with *T. brucei*, large numbers of macrophages and polymorphonuclear leukocytes are found in sites of tissue injury. Both cell types are capable of phagocytosing antigen–antibody complexes. Indeed, complement fixation by immune complexes is one of the mechanisms by which polymorphonuclear leukocytes may be attracted into sites of inflammation, the best example of this being the Arthus reaction. Furthermore, during phagocytosis of immune complexes by polymorphonuclear leukocytes, there is leakage of enzymes from their granules into the surroundings (Henson 1971; Weissmann et al. 1971). If sufficient enzymes are released, either during phagocytosis or from dying polymorphonuclear leukocytes, they can be cytolytic to tissue cells.

It has been shown recently that trypanosomes or their variant surface antigen are capable of activating complement by the conventional pathway, in the absence of antibody (Musoke and Barbet 1977; Nielsen and Sheppard 1977). This mechanism may contribute to the hypocomplementemia observed in infected animals (reviewed by Assoku, Tizard, and Nielsen 1977), although its relative importance compared to fixation of complement by immune complexes remains to be determined. Whatever causes the decrease in available complement, the effect is likely to favour the trypanosome by lessening the efficiency of antibody-complement-mediated lysis of trypanosomes and possibly by reducing the ability of macrophages to phagocytose by means of the C3 receptor.

Influence of Tissue Lesions on the Lymph Nodes

In addition to causing tissue injury, the presence of *T. brucei* organisms within the tissues has profound effects on the drainage lymph nodes. The cellular changes in lymph nodes are greatly influenced by the amount and type of antigenic challenge presented to the nodes in the afferent lymph. In dogs infected with *T. brucei*, trypanosomes can be seen in the lymph node sinuses from about 2

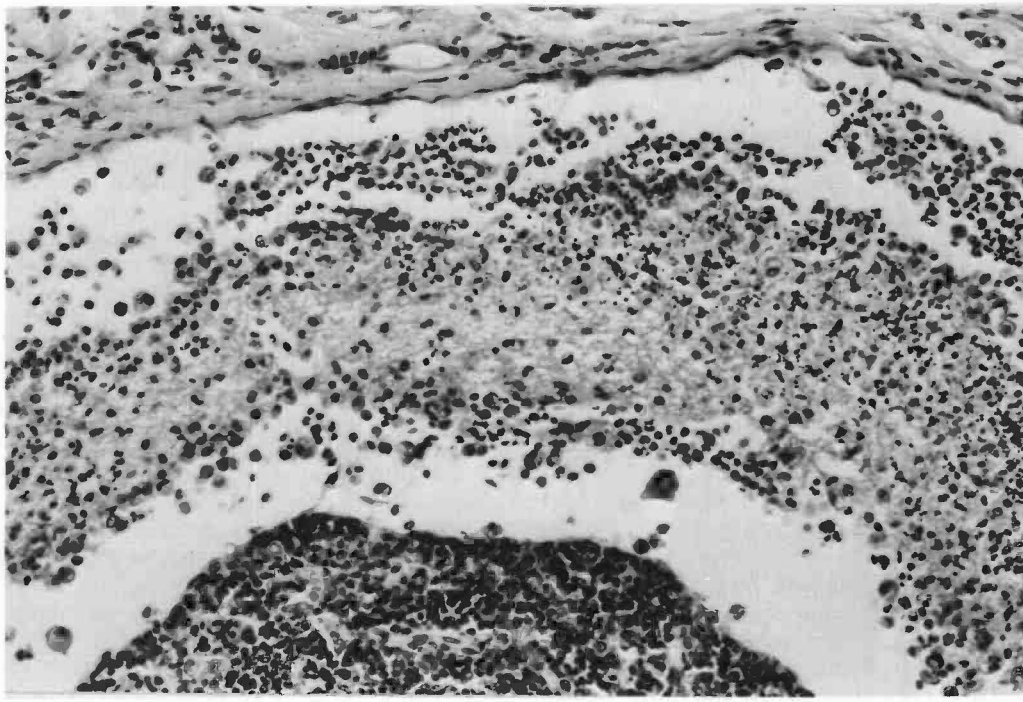

Fig. 4. *Lymph node of dog examined 24 days after inoculation with* T. brucei. *There is distension of the subcapsular sinus, which contains numerous macrophages, polymorphonuclear leukocytes, and cell debris within a network of fibrin. There is no longer a distinguishable fixed network of phagocytic cells in the sinus.*

weeks post infection. At 2 weeks, the sinuses also contain numerous lymphoid cells, many of them lymphoblasts, indicating active traffic of lymphocytes through the tissues. There is also widespread proliferation in the lymph node cortices, and large numbers of lymphoblasts and plasma cells are present in the medullary cords. From about 3 weeks post infection, the picture in the lymph nodes is much different. The cortices are much narrower and show less active proliferation; the number of lymphoid cells in the sinuses is much reduced; and the sinuses are distended and contain large numbers of macrophages and polymorphonuclear leukocytes. As in peripheral lymphatics, a mixture of these two cell types is found in the subcapsular sinuses; fibrinous thrombi similar to those observed in the lymphatics are also present (Fig. 4). By contrast, the medullary sinuses contain mainly macrophages, which show abundant eosinophilic vacuolated cytoplasm and sometimes contain granular phagocytosed material. A striking feature of the sinuses is the disruption of the normal fixed network of phagocytic cells; the majority of the macrophages present in the sinuses are rounded and show no attachment to the sinus lining.

Thus, in terminal cases of *T. brucei* infection in the dog, there appears to be a depletion of the lymph node cortices, a reduction in traffic of lymphoid cells entering in afferent lymph from the tissues, an accumulation of macrophages in the sinuses, and disruption of the normal sinus phagocytic network. These changes undoubtedly arise partly from the severe tissue lesions and the resultant drainage of trypanosomes, cells, and large quantities of antigen via afferent lymphatics into the lymph nodes. Ultimately, because of the severe alterations in the lymph nodes, they may no longer be able to respond adequately to antigenic challenge; this may be particularly important with regard to the appearance of new variable-antigen-type trypanosomes within the tissues.

Tissue Lesions in Trypanosome-Infected Cattle

In cattle infected with *T. brucei*, the heart is one of the most consistently affected organs. The lesions are similar to, but less severe than, those found in the dog and must be considered as functionally significant. In cattle, *T. congolense* and *T. vivax* also produce myocardial damage which is fairly distinctive for the organism involved. With *T. congolense*, perivascular and interstitial edema usually predominates and is accompanied by a scanty infiltrate of small lymphocytes and macrophages. In cattle infected with *T. vivax*, the cellular infiltrate, which is predominated by small lymphocytes, can be intense and, as with *T. brucei*, the organisms may be found in extravascular locations. In the bovine, irrespective of the species of trypanosome, myocardial necrosis is rare, but degenerative changes may be severe and extensive.

Organ and tissue weights in diseases caused by *T. vivax* and *T. congolense*

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Abstract. This paper summarizes observations on two large experiments that were undertaken to compare the diseases caused by *T. congolense* and *T. vivax* in cattle. The results are presented primarily to complement the descriptions of the histologic lesions discussed in Valli, Forsberg, and Mills p. 179 and Mwambu and Losos p. 184.

The lack of pathognomonic lesions in tissues and organs of animals infected with *T. congolense* and *T. vivax* makes a diagnosis on the basis of postmortem examination impossible and creates a problem in establishing major mechanisms other than anemia involved in the pathogenesis of the two syndromes. To obtain a complete picture of the changes that occur in tissues and organs, one must relate the histological and ultrastructural changes to the weight and size of the organs and tissues. Only a complete description of the structural change of tissues provides an indication of the functional alterations that are caused by the trypanosomes.

Materials and Methods

Because of the variability in the severity of responses that have been observed in uniform groups of cattle to a single infection, large numbers of animals are needed for studies of the different clinical syndromes. Using large populations enables investigators to measure the change in organ weights and to determine statistically significant differences. In our study, 10 Boran cattle were examined during early and late stages of disease.

Results

Tissue and organ weights of animals with *T. vivax* and *T. congolense* infections of 7 days duration were compared; and the findings indicated no significant differences between their lymph nodes and thymus and those of controls. However, the spleens were already heavier by 50%. An increase in weight was also observed in the liver (25%) and kidneys (60%) in both infections. Of particular interest was a significant increase in the weight of the heart (15%) in *T. congolense* infection.

When the tissue and organ weights in animals with *T. congolense* and *T. vivax* infections of 60 days duration were compared, an increase in the weights of lymph nodes, up to 50%, in *T. vivax* infections was found, but no increase was observed in *T. congolense*; in fact, there were indications that the nodes were smaller than those of controls. In all animals, the spleens were enlarged by 50% and the thymuses were greatly reduced (to about 30% of those observed in controls). Significant increases in weights were observed in the following organs: the adrenals 25%, the liver 50%, the heart 15%, and the kidneys 25%.

In summary, significant changes in weights of tissues and organs occur both early and late in infections and must be taken into consideration when evaluating the histological changes.

Discussion

In the literature, references have been made to the changes in lymph nodes and the spleen, the latter recorded as enlarged, fibrotic, or atrophied in various forms of trypanosomiasis. In our controlled experiments, we undertook statistical analysis and concluded that the size and weights of various organs and tissues depend on the species of trypanosomes that are causing infection and on the severity and duration of the disease syndromes.

Pathology of *T. congolense* in calves

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Abstract. Holstein calves injected with the TREU 112 strain of *T. congolense* developed a chronic debilitating disease characterized by anemia, pyrexia, intermittent diarrhea, and poor weight gain. Pancytopenia persisted throughout the period of observation and fibrinogen levels were reduced and turnover increased. Pathologically there was thymic atrophy with enlargement of the heart, lungs, liver, spleen, kidneys, and lymph nodes. The contents of the small intestines were increased in weight and there was red marrow in the midfemoral shaft. Histologically there was widespread microvascular dilatation with decreased cellularity in all lymphoid tissues and thymic-dependent atrophy. Chronic mononuclear inflammatory foci were present in the heart, liver, and kidneys, and renal glomeruli were diffusely enlarged and hypercellular. Hemosiderin deposition was prominent in the lungs, liver, spleen, renal epithelium, and bone marrow.

The principal lesion caused by *T. congolense* infection in cattle has been described as dilatation of capillaries in all organs of the body (Losos et al. 1973; Fiennes 1950). The strictly intravascular habitat of *T. congolense* has been described by Fiennes (1946), although most pathologic descriptions appear to include changes due to mixed infections and due to autolysis.

Anemia has been recognized as an important symptom of *T. congolense* infection and has been quantified by modern methods (Maxie, Losos, and Tabel 1976). It appears to be hemolytic and complicated by terminal hypoproliferation and vascular dilation and likely sludging (Valli, Forsberg, and McSherry 1978). The mechanism of this dilation has not been identified, although it may be similar to the kallikrein activation in bovine babesiosis (Wright 1973) and mediated by material released by intravascular trypanosomes (Tizard and Holmes 1977).

Materials and Methods

Twenty-four castrated Holstein calves were used in our experiment. They were 3–5 months of age and weighed 80–100 kg at the beginning of the experiment. Thirteen calves were given 1×10^6 *T. congolense* TREU 112 intravenously and were observed for the next 6 months. Hematologic studies were carried out as well as a variety of coagulation tests, and the animals were weighed weekly. Necropsy procedures consisted of inducing recumbency by intravenously injecting chloral hydrate and killing by electrocution. Necropsy was done immediately.

Results

Animals infected with *T. congolense* had persistent, moderate pyrexia and gained weight more slowly than did control animals. Following infection, they experienced a drop in hemoglobin value (initial mean, 11 mg/dl; mean at 16 days post infection, 7.8 mg/dl), and the red cell count continued to decrease to a low of $5.2 \times 10^6/\mu l$. On the 23rd day of infection, it was stabilized by a macrocytic response that was normocytic and almost fully saturated. During the next 6 months, the infected calves remained significantly but not critically anemic. The red cell life span decreased to about half that of the control animals, and the marrow myeloid-to-erythroid ratio changed from a normal of 1:2 to less than 1:5 in the infected calves (Valli, Forsberg, and McSherry 1978). There was a mild reduction in red cell mass and by indirect measurement an apparent increase in plasma volume in the infected calves.

Platelet levels in infected calves were about half those of the control animals but seldom dropped below $10^5/\mu l$ and were never sufficiently

decreased in individual animals to allow purpuric hemorrhage. Fibrinogen levels were decreased below those of control animals after the 10th day of infection and remained at about 400 mg/dl, half the normal level for cattle. Ethanol gelation tests were consistently negative in infected and control animals, and partial thromboplastin times were only irregularly prolonged in infected calves. Clot retraction tests were carried out, and the mean percentage clot retraction test for infected animals was not significantly different from that of the controls. Platelet kinetics were determined by ^{35}S methionine, and the times were not significantly different. The percent utilization of isotope in the platelet mass, however, was reduced in infected calves, indicating ineffective thrombopoiesis. The mean time for fibrinogen survival determined by ^{35}S methionine labeling was 7.6 days in infected calves and 12 days in control calves, and the difference was significant. The fibrinogen turnover rate and percent utilization of isotope both were higher in infected calves, indicating that the reduction in fibrinogen was a consumptive change.

Pathological Changes

Infected calves had pendulous abdomens because of a greater quantity of fluid in the small intestine. They had much less body fat and body flesh and a greater proportion of viscera to carcass weight. Significant changes in the thoracic viscera indicated a marked atrophy of the thymus gland of infected calves that weighed 0.21% of body weight as compared to 0.35% for control calves (Valli, Forsberg, and Robinson 1978). The hearts from the infected calves were more globose and tended to have less fat around coronary vessels; they weighed 0.67% of body weight, significantly higher than the 0.53% for control calves. Lungs from infected calves did not collapse completely, had a rubbery texture, and were tan; they weighed 1.5% of body weight compared to 1.2% of body weight for control animals.

The liver was grossly enlarged in infected calves and weighed 2.69% of body weight; the mean for the control calves was 1.67%. The livers were dark red and had a faint lobular pattern on cut surface and fine white striations extending out from the larger vessels into the lobular parenchyma. Livers of infected calves had a greatly increased resistance to digital pressure, and when placed on a necropsy table with the diaphragmatic surface down, the edges remained elevated from the surface. The spleens were large and significantly heavier in infected calves (mean of 0.47% of body weight). Due to the induction of anesthesia in calves before electrocution, the spleens of control animals were congested and oozed blood from the cut surface after removal. In contrast, the parenchyma of the spleens from infected calves were dry, and the organs were turgid, maintaining their shape when placed on the table. The cut surface of the spleen had a dark sinus area and prominent white lymphoid follicles.

The kidneys of all the calves were normal in outline but heavier in infected calves with a mean of 0.53% body weight, compared to 0.39% of body weight for control calves. The external capsule and cut surface of the infected animals' kidneys had the same tan discoloration as the lung. All body nodes were enlarged, although they were not weighed. The medullae were discoloured with reddish-brown striations extending deep into the subcapsular areas. Hemal lymph nodes became prominent in the flank areas after several weeks of infection and were especially prominent in the abdominal cavity, often forming a continuous chain from the renal node back to the iliac nodes at the bifurcation of the aorta. These structures were spherical, from 0.5 to 1 cm in diameter, and dark red on cut surface. The most prominent change in the hematopoietic system was marked conversion of fat to hematopoiesis in femoral marrow with almost all but a small central area of medullary fat converted to hematopoiesis in most animals (Valli, Forsberg, and Robinson 1978).

Histological Changes

The changes in the hematopoietic organs of infected calves constitute a remarkable spectrum of stimulation and depletion. Thymic lobules were smaller than those in control animals, and there was a prominent reduction in cortical width. Interlobular septa were infiltrated with fat and frequently eosinophils, and there was a noticeable reduction in density of lymphocytes in both the cortex and medullary areas. Furthermore, lymphocytes that were present in the cortex changed in mean size from small (with a nuclear diameter slightly larger than that of a red cell) to medium (with a nuclear diameter close to two red cells) (Valli and Forsberg in press).

The changes in lymph nodes were similar in all body sites and consisted of follicular hyperplasia with the presence of follicles in the medullary areas and a consistent and marked atrophy of thymic-dependent areas,which in some cases extended through to the capsule and left the follicles without a normal cuff of small lymphocytes. The follicles

of infected calves were generally larger than those in control animals, though the cell density within the germinal centres themselves was lower, and in many cases there was follicular hyalinosis and surprisingly fewer mitoses in infected animals than in controls. The areas normally occupied by thymic-dependent small lymphocytes had a marked increase in stromal collagen and a dense collection of large macrophages, often with prominent hemosiderin accumulations. Focal plasmacytosis occurred around small vessels in the medullae, and the postcapillary venules were prominent with high vesicular endothelium. The follicular changes were similar in the spleen with relative lymphocytic depletion in both follicular and thymic-dependent areas, but there was a marked increase in fixed cells in the sinus areas, many of which were hemosiderin-bearing macrophages.

The liver changes consisted of hepatocellular atrophy with sinusoidal dilation and endothelial and Kupffer-cell proliferation. Periportal lymphoid accumulations occurred but were not prominent, and no areas of necrosis were present.

Renal changes consisted of lymphoid aggregates occasionally including germinal centres around arcuate arterioles and occasionally in juxta glomerular locations. There was a moderate overall atrophy of tubular epithelium without areas of necrosis and a generalized increase in glomerular size and mesangial density. Hemosiderin stainable with Prussian blue was present in basilar areas of proximal convoluted tubules and within the mesangial areas of the glomeruli. There was a diffuse and global increase in glomerular cellularity, and luminal area was decreased occasionally with hemosiderin-bearing macrophages within capillaries. Synechiae frequently occurred between the visceral and parietal layers of Bowman's capsule; otherwise the parietal capsules were of normal thickness.

Consistent mononuclear infiltrates occurred within the myocardium, and there was an overall increase in numbers of myocardial nuclei per unit area. Nuclei were larger and more vesicular than in control animals.

Pulmonary changes consisted of a marked and consistent increase in width of alveolar walls in all areas of the lung. There were increased numbers of inflammatory cells within the alveolar walls and stainable iron was present throughout the alveolar septa in all infected calves. There were more inflammatory cells in the alveoli of infected calves but peribronchiolar lymphoid cuffs were not prominent. Trypanosomes could occasionally be seen within the pulmonary capillaries.

The adrenal gland had an overall increase in cortical width, which was largely due to an increase in width of the zona fasciculata. Nuclear density was greater in the zona fasciculata indicating that the cells within, although more numerous, were smaller than those of control animals.

Changes in the brains of infected animals were not marked and consisted of a mild dilatation of the perivascular spaces and increased numbers of lymphocytes in these areas. The most marked change was a uniform dilation of capillary lumina, many of which contained hemosiderin-bearing macrophages apparently fixed in situ. Trypanosomes were frequently seen in these areas and were most numerous in the vessels of the corpora quadrigemina.

Pituitary glands contained increased numbers of sinusoidal inflammatory cells and occasional actual germinal centres. Nuclear density was greater in the infected animals, indicating some reduction in the size of the pituitary cells.

Changes in the enteric tract consisted of a thickening of the squamous epithelium of ruminal villi in both the germinal and keratinized layers. There was mild reduction in thickness of glandular mucosa in the abomasum of infected calves and a shortening of villi in the small intestine. A greater area of lamina propria in the intestinal villi of infected calves was occupied by eosinophils and plasma cells. In addition there was a mild and consistent dilation of the central lymphatics of the intestinal villi. There was a mild reduction in pancreatic acinar cell size and in cellular area occupied by zymogen granules.

The bone marrow of infected calves was much more cellular than that of normal animals, generally 70–95% cellular as against 10–20% in control animals, with the remaining area being occupied by lipocytes. The fat cells in infected calf marrows were only half the size of those in control animals. Roughly 5 times as many megakaryocytes were found per unit area of marrow in infected calves as were found in controls, and these cells were roughly equal in diameter, although the nuclei of the infected calves were larger. Lymphoid nodules (and occasionally germinal centres) were frequently present in the marrow of infected calves and were never seen in the marrow of control animals. Hemosiderin was increased in marrow of infected calves and tended to be larger and more coarse than in controls. Plasma cells were much more numerous in marrow of infected calves as were vascular endothelial cells — a finding that indicates a mild degree of myelofibrosis. There were approximately three times as many myeloid cells per unit area in infected calves and about 15 times as many erythroid cells. Red blood cells were 6 times as numerous per unit area in the marrows of infected calves, indicating capillary dilation in this tissue.

The omentum of infected calves had red discoloured areas that did not blanch on digital pressure. Histologically these foci were due to capillary and venular dilatations with a marked increase in stromal pericytes and small lymphocytes.

Discussion

Changes in the tissues of calves with trypanosomiasis are in many ways like those of horses with equine infectious anemia where Jubb (1970) noted that in individual organs the lesions themselves were not specific but, taken collectively, produced a spectrum of change not seen in any other disease. *T. congolense* in calves caused anemia and neutropenia followed by a degree of recovery, the sequence resulting in generalized reticuloendothelial hyperplasia. This change is remarkable in that it results in a gross increase in volume of tissue in node and spleen and marrow at the expense of a reduction in cell density. The atrophy begins in the thymus and moves concurrently to the thymic-dependent areas of lymphoid tissues throughout the body. Although germinal centres are larger in infected calves, their cellular density is lower, and, most significantly, the number of mitoses per unit area in these areas is lower than in control calves, indicating incipient lymphoid depletion.

The outcome of *T. congolense* infection in calves appears to reflect the rapidity with which marrow hyperplasia occurs. In fulminating diseases, the calf responds poorly when marked increase in demand is made on both myeloid and erythroid marrow production (Valli, Forsberg, and Lumsden in press). The major differences between the results from Guelph, Canada, and those from Muguga, Kenya, are that the infected calves at Guelph developed marked marrow hypertrophy with extension of red marrow throughout most of the medullary cavity, whereas those in Kenya did not. Neutropenia was more severe in the African animals, and death often resulted from secondary bacterial infection. These differences may be due to age: the animals in Guelph were younger and may have been able to reactivate their neonatal hematopoietic sites with greater rapidity than were the more mature animals.

Vascular dilation is a consistent and remarkable finding in *T. congolense* infection and involves virtually all organs. The parasites themselves are only found with any regularity in the brain and skeletal muscle and occasionally in lung and myocardium if the parasitemia is high. The intravascular parasites undoubtedly interfere with circulation and are responsible for the rapid onset of depression and incoordination in animals dying of infection. The reduction in level of plasma fibrinogen with shortened life span and the increased uptake of isotope indicate a consumptive error associated in fibrinogen kinetics. Surprisingly, platelets are not consumed at a greater rate, but their reduced numbers in the peripheral blood with normal life spans are associated with an increased marrow megakaryocytic mass with increased nuclear size and reduced cytoplasmic volume, indicating ineffective thrombopoiesis. It is likely that these changes are accelerated when infection is acute rather than chronic, prompting a full-blown consumption coagulopathy. Whether infection due to *T. congolense* will be peracute or chronic is related to each animal's resistance, its nutritional plane, its freedom from other diseases, and the age at which it is infected (Forsberg et al. in press).

The two most productive areas for future research appear to be hemodynamics and the mechanisms by which the organisms produce microvascular dilation. In addition to the increase in size of hearts in infected animals, there is an increase in width of the media of the muscular arterioles of the lung and of the kidney and increased prominence of the juxta glomerular cells of the renal glomeruli, all suggestive of both pulmonary and systemic hypertension. Anemia, which is hemolytic and initially responsive, is seldom severe enough to be life threatening but appears to be complicated by vascular dilation, which is likely an adaptive mechanism to increase flow through areas obstructed by intraluminal parasites and marginated red cells.

The homing of the trypanosomes to brain and skeletal muscle is remarkable and likely related to endothelial changes in these organs. There are regional differences in the mitochondria of endothelial cells throughout the body, and this may interact with the energy system of the parasite such that it finds some areas more conducive to growth than others. ^{51}Cr-labeled trypanosomes given to normal calves and calves that had been infected and were sterilized with Berenil caused anaphylactic reactions in the previously infected calves and the sludging of parasites in the lungs of all animals. These changes are likely hemodynamic and related to jugular vein injection and not an indication of the parasite-homing process. The mechanism by which the parasites adhere to the endothelium is unclear and deserving of further study. Macromolecular compounds such as dextran, which will sweep sequestered red cells back into axial flow, have no effect on parasitemia due to *T. congolense*. Thus the reaction does not appear to be due to electrical

charge on the parasites. Similarly heparin, which has an antisludging effect on red cells has no effect on the attachment of parasites to vascular endothelium. Berenil rapidly releases the organisms into axial flow where they are still motile in wet mounts. The parasitemia is transient, and apparently the free organisms are rapidly removed by the reticuloendothelial system. In contrast, reticuloendothelial blockade with corticosteroids causes a pronounced and continued increase in parasitemia likely due to a reduction in phagocytic removal. The effects of the organism on the host cannot be explained simply as a toxic phenomenon, because young calves infected with *T. congolense* occasionally develop very high parasitemias of 10 000–80 000/μl of blood during the first 10 days of infection and are completely asymptomatic. They may very suddenly become depressed and recumbent associated with a reduction in parasitemia. It is tempting to speculate that this reduction in parasitemia is coincident with the appearance of antibody that causes the parasite to home in on muscle and brain and, thus, to produce cerebrovascular blockage and functional depression.

In summary, it seems that the study of the kinin system in calves infected with *T. congolense* should be undertaken along with an intensive, hemodynamic workup on the animal dying of the uncomplicated disease.

Ultrastructural changes in blood vessels of tissues of cattle experimentally infected with *Trypanosoma congolense* and *T. vivax*: a preliminary report

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Abstract. One-micron sections (stained with toluidine blue-O) of kidney, heart ventricular muscle, and brain collected at post mortem from experimentally infected cattle, were examined by a light microscope. Marked changes were observed. These involved enlargement of the kidney glomeruli with marked dilation of the glomerular blood capillaries in *T. congolense*-infected animals. The changes were less marked in *T. vivax* cases and absent in the controls. There were large mononuclear cells in the dilated glomerular capillaries in *T. congolense* sections — a finding that was less marked in *T. vivax* cases and absent in controls. Blood capillaries between heart muscle fibres were dilated and patent in *T. congolense*, but not in *T. vivax* and controls. In brain sections, a marked dilation of the brain capillaries, with an apparent thickening of the endothelial wall and signs of edema were observed in *T. congolense* and in *T. vivax* cases, but not in controls. Mononuclear cells similar to those seen in glomerular capillaries were seen in some brain capillaries. Apart from anemia, which is caused by both *T. congolense* and *T. vivax* infection in cattle, the dilation of the blood capillaries in the kidney glomeruli and heart muscle would likely lead to changes in the hemodynamics of the circulatory system. The changes would include a pooling of blood in the peripheral circulation and interference with the normal interchange of metabolites. The effect of pooling would be circulatory malfunction and, eventually, cardiac failure.

Trypanosomiasis in cattle is caused by *T. brucei*, *T. congolense*, and *T. vivax*, all of which present different pathogeneses. In *T. brucei* infections, the parasites have been found to localize mainly in subcutaneous and connective tissues (Losos and Ikede 1972). The result is inflammation and mononuclear cell infiltration, which gives rise to lesions in the eyes and urticarial-like lesions. In *T. congolense* and *T. vivax* infections, on the other hand, the parasites localize primarily in blood vessels, the *T. congolense* parasites affecting especially microcirculation, with either few or no obvious clinical signs other than high temperatures at peak parasitemia and accompanying anemia. Because of the lack of pathognomonic signs and lesions, it is difficult to differentiate between *T. congolense* and *T. vivax* infections. Furthermore, it is difficult to say how the parasites cause tissue damage.

The gross pathological lesions caused by *T. brucei* in connective tissues are relatively easy to describe, but gross lesions caused by *T. congolense* and *T. vivax* are difficult to detect. Occasionally, there are hemorrhages seen in acute *T. vivax* infections and serous atrophy of fat and extensive edema of different organs in severe *T. congolense* infections.

T. congolense attaches to the endothelium of blood vessels (Banks 1978). It is possible that this attachment leads to changes in the functioning of these vessels, thereby affecting the normal interchange of fluids and metabolites between the vessels and the surrounding tissues. Our studies intended to test this hypothesis by carefully studying structural changes in the blood vessels that take place because the parasite is attached to the vessel endothelial wall. Because the changes are difficult to detect clearly under light microscopy, we decided to undertake ultrastructural studies of the vessels, with emphasis on the microcirculation, of selected tissues.

Because large populations of *T. vivax* are found

in circulating blood, their effect on the endothelium was also to be investigated.

Materials and Methods

Fifty comparably sized Boran steers, aged 10–12 months, were divided into three groups: two groups of 20 were infected with *T. congolense* and *T. vivax* respectively, and the remainder (10) acted as a control. The *T. congolense* and *T. vivax* groups were subdivided into two groups each to represent the early and late infection stages.

The steers were killed during high and low parasitemias (7 and 56 days post infection respectively), and postmortem examination was carried out.

Standard procedures were used at post mortem and in fixing of tissues for electron microscopy. After processing, the tissues were embedded in Beem capsules in an araldite-epoxy mixture and subsequently trimmed. One-micron sections were then cut and stained with 1% toluidine-blue-O in 3% boric acid. Some difficulty was experienced in obtaining well-stained sections, especially from brain and heart.

Results

Dramatic pathological changes had occurred in the 1-μm sections. In the kidneys of all the infected animals, the glomeruli were noticeably larger than those of the control animals.

The blood vessels in the glomeruli were markedly dilated in *T. congolense* infections and less so in *T. vivax* cases. The blood vessels of control cases showed no dilation.

There were many large mononuclear cells in the glomerular blood vessels in *T. congolense* cases, few if any in *T. vivax* cases, and not detected in control cases.

The blood capillaries between the heart muscle fibres were dilated and patent in *T. congolense* and *T. vivax* cases, and although they could be seen in the control cases, they were not enlarged.

In the brain, the blood vessels were not dilated in controls, but in *T. vivax* and *T. congolense* cases, they were markedly dilated. There was an apparent thickening of the endothelial wall and signs of edema. Mononuclear cells similar to those seen in the glomerular blood capillaries were found in some brain vessels.

Discussion and Conclusions

The results indicate that in *T. congolense* infections, marked changes occur in microcirculation. The extensive dilation of the capillary bed in the brain, heart muscle, and kidney glomeruli suggests a change in the hemodynamics of the circulatory system and, in turn, a pooling of blood in the capillary bed. The pooling is likely to affect dramatically the efficiency of circulation through the capillaries.

Even at 500 times magnification, the 1-μm sections of tissues of *T. congolense*-infected animals show changes in capillary walls, which appear thickened and show evidence of perivascular edema. Comparable changes have not been detected in *T. vivax* sections, except from the brain. In fact, the differences between *T. congolense, T. vivax*, and control tissues are so dramatic that the three conditions can readily be distinguished.

Although anemia is a sign of both *T. congolense* and *T. vivax* infections, further malfunctions of the blood capillaries seem to accompany *T. congolense* infections.

The combination of anemia and increased peripheral volume could overwork the heart and eventually lead to myocarditis and cardiac failure. The pooling of blood in the capillary bed would interfere with normal circulation and, in turn, with the normal exchange of metabolites.

Discussion summary

V. Houba and G.J. Losos

The pathogenesis of trypanosomiasis is multifactorial: animals have fever, watery diarrhea, internal and external lesions, etc. On one hand, the lesions are similar to those observed in graft-versus-heart disease (GVH) in humans and other animals. In GVH, transplanted T-lymphocytes react against host antigens, whereas in trypanosomiasis, sensitized T-lymphocytes react against parasite antigens. On the other hand, the intestinal disturbances observed in trypanosomiasis resemble those caused by cholera toxin, which activates adenyl cyclase in the intestinal epithelial cells and leads to the disturbance in water and electrolyte transport.

The mechanisms responsible for the lesions in the disease are many; they include immune complexes, the activity of macrophages, the localization of the trypanosomes, etc. The role of immune complexes has not been clearly determined, but they appear to be responsible for at least some of the lesions. Living and/or dead trypanosomes release antigens that combine with antibodies to form immune complexes, which are normally removed by physiologic routes, i.e., the reticuloendothelial system. But if the immune complexes are formed in tissues as a local response to antigens released from the circulation, they may be more difficult for the body to remove, persisting in the tissues and producing lesions, for instance, in the heart and perhaps the choroid plexus. If the reticuloendothelial system becomes overloaded, the immune complexes may also persist in the circulation for long periods, then localize in filtering systems, such as the glomeruli in the kidney, and cause the glomerulonephritis observed in experimental models of trypanosomiasis.

The immune complexes may also play a part in the activation of macrophages, which themselves can cause tissue lesions. Once activated, the macrophages appear to collect thromboplastin on their plasma membranes and, hence, may be responsible for consumption coagulopathy (diffuse intravascular coagulation), a feature of trypanosomiasis.

Lesions caused by trypanosomes that localize in tissues are different from those caused by bloodstream forms; the lesions caused by *T. vivax*, which is mainly found in the circulation, differ markedly from those caused by *T. brucei*, which localizes in the tissues. How tissue localization affects chemotherapy is not at present understood; however the diamidines have been shown to concentrate more in tissues than in blood and to vary in concentration among tissues.

As early as 1912, Wolbach and Binger in Guelph, Canada, and later (1928) Peruzzi described the lesions and tissue distributions of *T. brucei* in rats, rabbits, and monkeys. The trapping and engulfing of parasites by macrophages were observed in the rabbit's ear chambers by Goodwin in 1971, and occlusion of small blood vessels by accumulated macrophages and mononuclear cells was described.

The trypanosome revisited: a summary of the conference

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This has been an excellent conference — friendly, useful, and marked by the effective exchange of information. To sum it up and to do justice to more than 30 papers and the discussions that followed them are an impossible task. So I shall tell you a story — a fantasy, you may think — that has come from what I have heard during the last 3 days.

Each trypanosome in the tsetse fly prepares for action by whipping out of its flagellar pocket 7 million glycoprotein molecules and slipping them smoothly over its skin to the tip of its flagellum before it is shot down the tsetse's proboscis with a welter of saliva into the lush, warm, but dangerous environment of its new host. The glycoprotein garment is continually renewed like a creeping film, the spare bits being slashed off in redundant plasmanemes. The garment resembles a grassy field; almost all the glycoprotein blades belong to a single species, although there are a few shafts of related species here and there or an undergrowth of the shorter blades of cross-reacting determinants.

The trypanosomes slice themselves in half — multiply — and the new versions are clad in different shirts. Professor Vickerman thinks they differ from the beginning; Dr Jenni believes that they start the same and change later on. In any event, they can, and do, change *even* in vitro, and several or, perhaps, many separate populations of identifiable variants coexist. How they manage to change and how many changes are possible we do not yet know, but Drs Cross and Williams think that transfer of genetic material and measurement of its expression in bacteria will soon tell us the answer.

Before long, because of its more rapid growth, one variant outstrips the others and comes to form the major part of the population. Metabolism is intense and the constant traffic in the flagellar pocket — inflow of nutrients and outflow of glycoproteins and possibly injurious waste products — boggles the mind.

Meanwhile, the hosts' defences have not been idle; the expected antibody response mows down the first parasitemic wave and then all hell breaks loose. As Ted Valli puts it, "disease begins with the immune response." Complement is activated, perhaps by a hexose-containing factor from the trypanosomes, and certainly by immune-complex formation. Pieces of shed glycoprotein attach themselves to red cells, which are then gobbled up by the host's expanded mononuclear phagocytic system. Anemia ensues.

Trypanosomes killed by antibody autolyze and release Ian Tizard's dangerous cocktail of hemolytic, mitogenic, and immunosuppressive fatty acids. The body temperature rises. Hageman factor is activated by immune complexes and releases kallikreins and kinins; plasminogen is activated and serotonin and fibrinogen degradation products appear. Whether they always do so or not, these processes can affect capillary permeability, platelet aggregation, and hemodynamics.

As time goes on, the upgrowth of successive trypanosome variants, each eliciting a new antibody response, and the increased peripheral demand for cells from the bone marrow strains the systems of the host. The heroic efforts of tissue phagocytes deal with the trypanosomes but also consume red cells, and hemosiderin, useless for further hemoglobin synthesis, accumulates.

B cell polyclonal proliferation manufactures "nonspecific" antibody that may be specific for unknown variants, and immunosuppression, disadvantageous for resisting intercurrent infections, occurs, possibly as an attempt by the host to control its B-lymphocytes with suppressor cells.

And now you must make up your own stories, depending on whether you are thinking of *T.*

brucei, *T. vivax*, *T. congolense*, cattle, goats, rabbits, or mice, acute or chronic infections, well-fed animals in laboratories, or paltry-fed animals on the hoof. But this is a marvelous story and is not all fantasy. It could not have been told 2 years ago, and it has been built up by research workers in many countries.

Clearly, there are some things that should be done at once. The capacity for antigenic variation must be determined and more experiments must be done on the antigenic status of the metacyclic trypanosomes. Without this vital information, further work on the design of antitrypanosomal vaccines may perhaps be wasted work.

It is also clear that the techniques used in different laboratories must be defined; for one group of workers to reproduce the experiments of another, they must follow exactly the methods used. Elementary but if forgotten, it leads to much useless argument.

Ted Valli's beautiful quantitative histopathology and his demonstration of trypanosomes in dilated brain capillaries must be followed up; much more will undoubtedly be done on the intricacies of the T- and B-lymphocytes, suppressor cells, and immune complexes to keep abreast of the rapidly advancing concepts and techniques of modern immunology.

The Permanent Secretary, when he kindly opened this conference, said that our work should have a practical purpose and should lead to the control of trypanosomiasis and the betterment of the livestock industry. I believe that the conference, by concentrating on a relatively narrow field and working it hard and well, may have helped toward this end.

But it is no use for all of us to stay in the lab all the time. We now have a reliable quantitative data base and modern techniques to assess the response of various breeds and stocks of domestic animals to various fly and environmental challenges. With our new, accumulated knowledge of what to look for and what to measure, we need to go back to the field and carry out operational research projects — carefully planned and sufficiently large to answer specific questions, such as what animals, including domesticated wild species, will do best in marginal areas in which tsetse bite, and if, how, and when they should be treated to give the best growth and health with the least trouble and expense.

This kind of study is not cheap; it is usually more expensive than laboratory projects because it has to continue and to be firmly supported for at least 5 and perhaps 10 years or more to get reliable answers.

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